Effects of soluble mediators from *Staphylococcus aureus* on gut-brain signalling and intestinal function

PhD thesis

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Summary

This thesis investigates whether gut-brain signalling and intestinal functions are altered by soluble mediators produced by the opportunistic pathogen *Staphylococcus aureus*. Afferent nerves are known to respond to a wide range of stimuli and bacterial infections are frequently associated with symptoms of afferent signalling. The possibility that intestinal afferents are directly activated by mediators released from bacteria has however not been previously studied.

We have addressed this question by recording afferent activity in response to supernatants from cultures of *S. aureus* (SSA) in an *ex-vivo* preparation of mouse distal small intestine and colon and found that SSA induced profound changes of afferent firing when they were perfused into the organ bath. Using a microbiological approach, we found that α-haemolysin and phenol-soluble modulins contributed to the initial excitation and delayed inhibition of spontaneous afferent discharge as well as mechanosensitivity, respectively. The latter was also particularly involved in SSA-induced changes of cell membrane permeability in primary neurons measured by propidium iodide fluorescence. Neurons also express receptors for phenol-soluble modulins and α-haemolysin. Serosal application of SSA was found to reduced colonic contractility and increased secretion in Ussing chamber experiments. The prosecretory effect was alleviated in the absence of α-haemolysin. In sharp contrast to the serosal application, luminal SSA only induced minor changes of intestinal afferent activity and failed to increase intestinal secretion.

The responses to SSA displayed a large variability and we found that host factors such as bacterial colonisation and previous inflammation affected the excitatory and inhibitory components of the response. Surprisingly, Inflammation induced by colonic instillation of TNBS did not change baseline characteristic of small intestinal afferent nerves.

These findings support the hypothesis that afferent nerves contribute to bacteria sensing which may play an important role in initiating and controlling the physiological response of the host to bacteria. Future studies should aim to better understand the mechanisms underlying afferent activation by SSA and the physiological impacts.

Publications and presentations

The research performed as part of this thesis has been presented orally or in poster format at the following conferences:

Poster

FMN 2016 (26th-28th August, San Francisco, USA)

ANGMA Annual Meeting 2017 (31st March to 1st April, Melbourne, Australia)

NeuroGastro 2017 (24th-26th August, Cork, Ireland)

Oral presentations

Florey symposium 2016 (24th June, Sheffield, UK):
"Modulation of Gut-Brain Axis by Supernatants of *Staphylococcus aureus*"

APS Meeting 2017 (11th April, Adelaide, Australia):
"Modulation of Visceral Sensitivity by Bacterial Mediators produced by *Staphylococcus aureus*"

Abbreviations

5-HT	serotonin
ACh	acetylcholine
ADAM	A Disintegrin and metalloproteinase domain-containing protein
Agr	accessory gene regulator
AMP	anti-microbial peptide
ATP	adenosine triphosphate
CAP	cholinergic antiinflammatory pathway
CGRP	Calcitonin gene-related peptide
CNS	central nervous system
DRG	dorsal root ganglion
ENS	enteric nervous system
GIT	gastrointestinal tract
GPCR	G protein-coupled receptor
HC	healthy control
Hla	α-haemolysin
IBS	Irritable Bowel syndrome
IFN	interferon
IL	interleukin
IPAN	intrinsic primary afferent
JAX	mice obtained from the Jackson laboratory
LPS	lipopolysaccharide
LS	lumbosacral DRG
MMC	migrating motor complex
NA	nerve activity
nF-κB	nuclear factor kB
NG	nodose ganglion
PCR	polymerase chain reaction
PFT	pore forming toxin
PI	propidium iodide
PNS	peripheral nervous system
Psm	phenol-soluble modulin
Pvl	Panton-Valentine leukocidin
QS	quorum sensing
RNA	ribonucleic acid
SA	*Staphylococcus aureus*
SAHMRI	South Australian Health and Medical Research Institute
SCFA	short chain fatty acid
SE	*S. aureus* enterotoxin
SP	substance P
SSA	supernatant from cultures of *S. aureus*
TCS	two-component system
TL	thoracolumbar DRG
TNF	tumour-necrosis factor
TRP	transient receptor potential
TSB	tryptic soy broth
TNBS	2,4,6-Trinitrobenzenesulfonic acid

CHAPTER I

General introduction

1 The GI tract and host-microbe interaction

The gastrointestinal tract is essential for human physiology. It is responsible for degrading and absorbing nutrients that supply energy for our survival, stores and expels waste products, contributes to the regulation of hormone levels and homeostasis. In addition, it has sensory and immune-regulatory functions that are important for brain-gut communication.

1.1 The healthy gut

The GI tract includes the oral cavity, oesophagus, stomach, duodenum, jejunum, ileum, caecum, colon, rectum and anus. Although each of these segments has distinct functions, all segments share a similar radial anatomy which includes an epithelial layer, mucosa, submucosa, circular and longitudinal muscle and segments distal of the stomach are covered with a layer of connective tissue (serosa). It is densely populated with neurons and fibres of the peripheral and enteric nervous system (Yoo and Mazmanian, 2017). The latter is organised in plexi in the submucosa and between the longitudinal and circular muscle layers (section 1.1.2, p 20). The GI lumen is highly colonised with bacteria and in fact, it harbours the largest reservoir of bacteria that is associated with humans with numbers of bacteria outnumbering human cells (Aagaard et al., 2013; Huttenhower et al., 2012). We are only beginning to understand the complexity of the symbiosis between commensal bacteria (microbiome) and the human/mammalian host but it is clear that the intestinal microbiome has essential functions in the digestive process, provides nutrients and contributes to the development of the immune and nervous system. In contrast, pathogenic bacteria can have detrimental effects on human physiology and cause severe acute and chronic disease (Huttenhower et al., 2012; Morrison and Collins, 2016; Pickard et al., 2017). This section aims to provide an overview on current knowledge on how commensal and pathogenic bacteria interact with the components of the gastrointestinal wall. It should be noted that defining what constitutes a commensal or beneficial bacterium remains challenging because commensal bacteria can have pathogenic potential when they are present in the wrong place (intracellularly, blood stream) and similarly, some potential pathogens can be found in the microbiome

of healthy individuals. Therefore, it is necessary to further investigate what discriminates pathogens and commensals (Artis, 2008; Tlaskalová-Hogenová et al., 2004). It is suggested for example that commensals cannot activate the immune system to the same extent as pathogens because they do not produce virulence factors (section 1.2.1, p 31) that enable tissue penetration and invasion of cells. In contrast, commensals are able to modulate immune responses and induce oral tolerance (Artis, 2008).

1.1.1 Composition of a healthy gut microbiome

Since the discovery that the gut contains an unexpectedly high density of bacteria, the intestinal microbiome has gained much interest and with the availability of next generation sequencing, our understanding particularly about the "who is there" has exponentially increased. In contrast, classical microbiological culturing and staining techniques did not enable to identify the bacteria in the GIT. Gram staining has traditionally been used to quantify and broadly classify bacteria. Gram-positive ($gram^+$) bacteria possess a thick cell wall composed of > 40 layers of peptidoglycan which incorporates the crystal violet dye of the gram stain. In contrast, $gram^-$ bacteria have a small peptidoglycan layer and do not retain crystal violet during washing[1] (Coico, 2005; Moyes et al., 2009). Using sequencing techniques, it has been found that the bacterial composition between individuals displays a large variability and research has aimed to understand how extrinsic and intrinsic factors including genotype, lifestyle and disease affected the composition of the microbiome (Duval et al., 2017; Foster et al., 2017; Holmes et al., 2017; Maier et al., 2018). Because of the large inter-individual variability, we still only have a limited understanding of what is to be considered a healthy microbiome. Some general aspects are summarised below.

(1) A healthy gut microbiota is characterised by a high diversity which enables adaptation to different conditions and exert many different functions. As measures of diversity, α-diversity describes variation within a sample and β-diversity compared variability between samples/subjects. An ecologically rich community thus is characterised by a high α-diversity (Lyte and Cryan, 2014).
(2) We still only have a limited understanding about the total richness of the microbiome (Falony et al., 2016; Kurokawa et al., 2007). Even in most large scale studies, (> 1000 participants), only about 300-400 genera are detected but extrapolations estimate the existence of over 750 genera. In addition, many of these genera remain to be isolated, identified and characterised.

[1] https://laboratoryinfo.com/gram-staining-principle-procedure-interpretation-and-animation/

(3) Bacteria of the gut microbiota predominantly constitute five phyla. Bacteroidetes and Firmicutes are present in large quantities while Proteobacteria, Actinobacteria and Verrucomicrobia are less abundant. Some genera of these phyla are present in large quantities and detected consistently in most individuals. They are considered a core microbiome (Arumugam et al., 2011; Falony et al., 2016; Gill et al., 2006). Other genera and particularly pathogenic bacteria are only present in small quantities (low gene count). Those can only be found in studies with sufficient sequencing depth.

(4) The composition of the gut microbiota is spatio- and tempero-specific i.e. it changes along the longitudinal axis of the intestine and with age. The changes along the longitudinal axis are dictated by the local environment including pH, osmolality, availability of energy sources and oxygen concentration. Therefore, the abundance of *Lactobacillus* for example is high in the proximal compared to the distal gut. The overall population density increases sharply towards distal regions and peaks in the colon were bacterial densities reach $10^{10\text{-}12}$ per gram (Stearns et al., 2011). Age-related changes of the gut microbiota are inter-related with changes of lifestyle throughout a person's lifespan (Scott et al., 2017; Yatsunenko et al., 2012). For example, *Staphylococcacae*, *Corynebacteracae* and *Propionibacteriacae* colonise the gut in children that were delivered via caesarean section whereas *Lactobacillacae* and *Prevotella* dominate the microbiota in naturally delivered babies. Later in life, breastfeeding increases *Lactobacillus*, *Staphylococcus*, *Enterococcus*, and Bifidobacterium and in adulthood exposure to environmental bacteria, food antigens, social interactions, etc. increases the abundance of various bacteria contributing to the high inter-individual variability (Bashiardes et al., 2018).

Analyses of this variability have led to the concept of enterotypes. Enterotypes are reproducible somewhat distinct patterns of bacteria composition that account for the variability of the microbiome. These patterns have been described in a number of studies and although these differ with regard to classification and sequencing techniques, it has been found that it is the abundance of the genera *Prevotella*, *Bacteroides* and *Ruminococcus* (Firmicutes) that is most variable between individuals. Therefore, their abundance, in combination with the abundance of some less abundant bacteria, can be used to classify humans into enterotypes (Arumugam et al., 2011; Costea et al., 2017). It yet remains to be determined however whether classification into enterotypes can be used as indicators for disease susceptibility (Nguyen et al., 2015).

Factors influencing the gut microbiome

The variability of the microbiome has prompted researchers to understand factors that contribute to this variability. These include intrinsic (genetics, stress) and extrinsic (diet, bacteria, xenobiotics) factors (Figure 1). The high interdependence of these factors is a major difficulty in the interpretation of human studies and makes it difficult to conclude about the effect of any one single aspect.

Diet and Prebiotics

The effect of diet on the composition of the microbiome is particularly complex and difficult to delineate because it is often intermingled with age, cultural habits and the geographical location. Furthermore, gut-residing microbes are functionally redundant which means that although the availability of energy sources and micronutrients will select for the growth of bacteria that are able to degrade specific substrates, it is hard to establish a causative relationship between dietary components and individual bacterial species (David et al., 2014a; Maukonen and Saarela, 2015).

The abundance of *Bacteroides* (phylum Bacteroidetes) for example is higher in humans that consume a protein-rich Western compared to a diet rich in complex carbohydrates. Species of this genus are particularly metabolically flexible and can switch between the fermentation of complex carbohydrates and proteins. By contrast, a high abundance of another Bacteroidetes genus, *Prevotella*, has been linked to the intake of carbohydrates (Bashiardes et al., 2018; Ha et al., 2014). It is associated with the microbiomes of African children (low-fat/animal protein; rich in starch, fibre and plant polysaccharides) compared to European children and a strict vegetarian's diet (Yatsunenko et al., 2012). Most other carbohydrate-associated bacteria such as *Ruminococcaceae* and *Lachnospiraceae* belong to the Firmicutes phylum (Korpela, 2018; Sonnenburg et al., 2010). Carbohydrates that are indigestible for the human host (prebiotics) including inulin, fructo- or galacto-oligo-saccharides ('FOS' and 'GOS'), increase *Bifidobacteria* (phylum Actinobacteria) and *Lactobacillus* (phylum Firmicutes) which may account for their health-promoting effects (Burokas et al., 2017a; Schroeder et al., 2017). However, fermentable carbohydrates (fermentable oligo- and disaccharides, monosaccharides and polyols, FODMAPs) have also been associated with to the development of chronic visceral pain (Zhou et al., 2018). This is supported by effective treatment of irritable bowel syndrome patients with a diet low in FODMAPs (Valeur et al., 2018). This diet reduces *Bifidobacterium* in stool samples and also decreases symptom severity (Bennet et al., 2017; McIntosh et al., 2017).

In contrast to carbohydrates and proteins (Holmes et al., 2017), much less is known about the effect of dietary fat on the microbiome. High-fat diets (HFD) have strong effects on microbiome composition (Murphy et al., 2015) but because they are most frequently, and particularly in animal models; linked

to obesity (see section 0, p 55), the effect of dietary fat on microbiome composition is difficult to delineate. Using a mouse model that is resistant to diet-induced obesity, Hamady et al. (2009) have described changes of HFD on microbiome independent from obesity. Consistent with the confounded human studies, they found that the *Bacteroidetes* are depleted under high-fat conditions and this was associated with an increase of bacterial stress-related genes (Hamady et al., 2009). The associated changes of bacterial metabolism and the increase of genes related to cell motility may also affect the epithelial barrier and thus, contribute to the increase of serum lipopolysaccharides after consumption of a high-fat meal (Erridge et al., 2007).

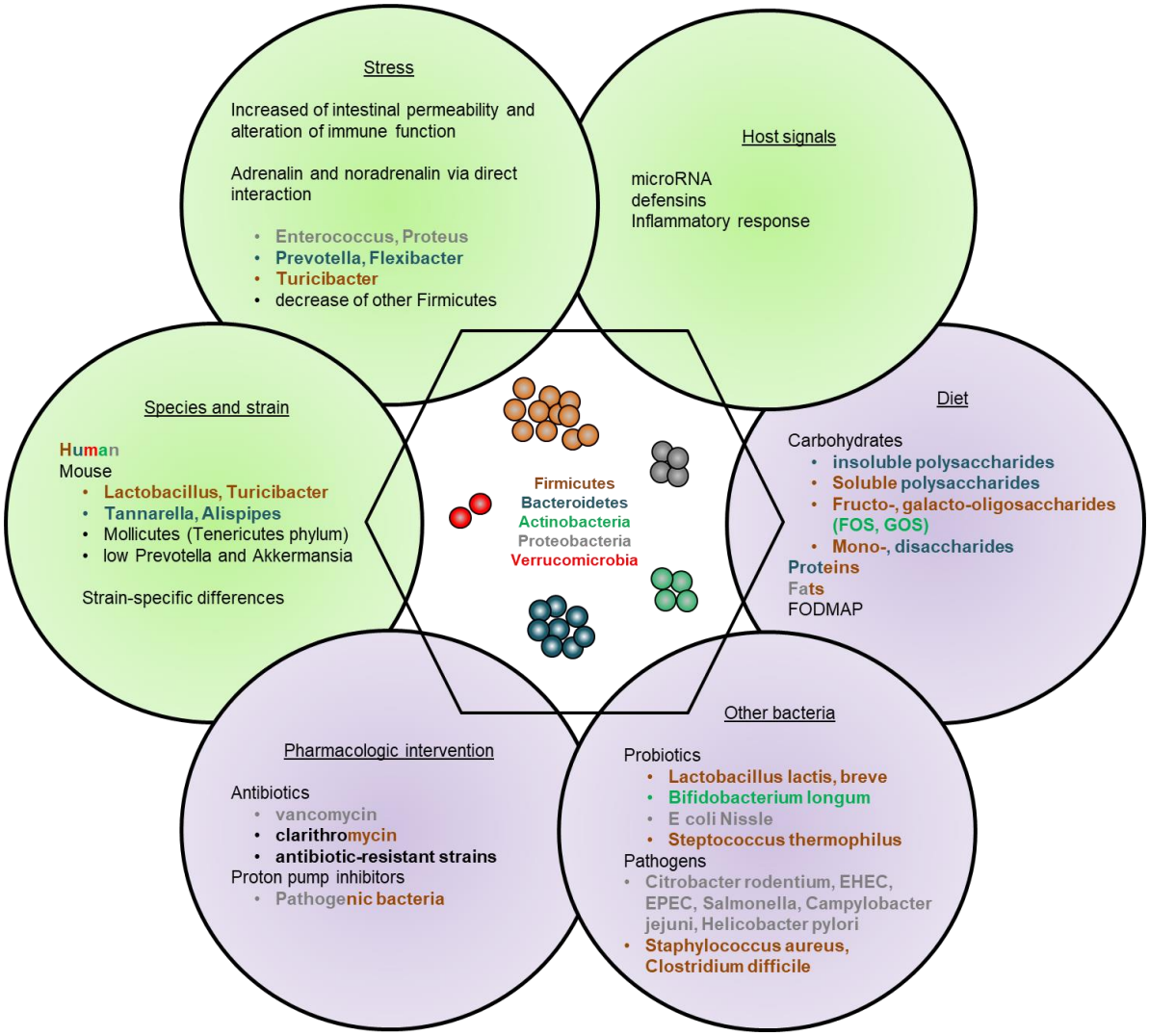


Figure 1: Summary of factors that contribute to the variability of the microbiome. Intrinsic factors (green) include species and strain, host signals and stress whereas diet, pharmaca and other bacteria constitute extrinsic factors (violet). Color code is used to refer to the phyla as indicated in the center. For details compare text below.

Bacteria (probiotics and pathobionts)

The high abundance and symbiotic relationship of commensal bacteria with their hosts (section 1.1.2) generally counteracts colonisation with environmental bacteria. However, food-related and environmental bacteria are present in the GI lumen and may, even though their presence is transient, affect the composition of the microbiome (David et al., 2014b).

It has been suggested that the health-promoting effects of probiotic bacteria which can be consumed with fermented food or supplied as capsules, are due to alterations of the microbiome. Indeed, administration of probiotics such as *Bifidobacterium* and *Lactobacillus* increases the stability of the microbiome and the abundance of the probiotic strain (Kajander et al., 2008; Larsen et al., 2006; Yoon et al., 2015). However, a number of studies also failed to detect profound changes of microbiome composition after probiotic treatment (BogoviČ MatijašiĆ et al., 2016; Kajander et al., 2007; Larsen et al., 2011). One explanation could be that probiotic species are also part of the healthy microbiome and thus, the effect of probiotic treatment strongly depends on microbiome composition prior to the intervention as well as the specific strain and the used amounts of bacteria. It could also be that it is their transient adherence to the intestinal epithelium which increases the epithelial barrier function or the production of antimicrobial substances and makes them more effective than commensal *Lactobacillus* or *Bifidobacterium* strains (Dunne et al., 2001; Jensen et al., 2012; Ren et al., 2014).

On the other hand, infection with pathogenic bacteria is thought to induce sustained changes of the microbiome. These bacteria are able to overcome colonisation resistance (section 1.1.2) because they express host-attachment proteins, antibacterial peptides and virulence factors that alter gene expression in the host and the commensal bacteria. For example, metabolites present in conditioned media from *Clostridium difficile* were shown to impair the growth of several strains of commensal bacteria (Horvat et al., 2017). Colonisation with *Salmonella enterica* or *Helicobacter hepaticus* required the virulence factor secretion system TTSS (type three secretion system) or cytolethal distending toxin (CDT) (Ge et al., 2005; Lam and Monack, 2014; Stecher et al., 2007) and *Citrobacter rodendium*, the mouse equivalent to pathogenic *Escherichia coli,* as well as *Staphylococcus aureus* express the attachment proteins translocated intimin receptor (Tir) and wall-teichoic acids which enable binding to host proteins (Deng et al., 2003; Misawa et al., 2015). The dependence on distinct factors suggests that pathogens could have specific effects on certain commensal bacteria and particularly affect those that compete with the pathogen. Although this might indeed be the case, it is often difficult to link infection to a specific pathogen and this suggests that infection-associated inflammation probably causes more profound

changes in the microbiome (Lupp et al., 2007; Pham and Lawley, 2014; Walker and Lawley, 2013). On the contrary, there is now good evidence that the presence of individual commensals can protect from infection with specific pathogens. A mix of *Lactobacillus grasseri*, *Parabacteroides goldsteinii*, *Muribaculum intestinale*, *Anaerostipes caccae*, *Clostridium* and *Bacteroides* species was found to be sufficient to decrease susceptibility to *Salmonella* colonisation and *Bacteroides sartorii*, *Parabacteroides distasonis*, *Clostridium bolteae* and *Blautia producta* were protective against vancomycin-resistant *Enterococcus faecium* infection (Caballero et al., 2017; Thiemann et al., 2017). This indicates that therapeutic targeting re-establishment of a healthy microbiome in addition to eradicating the intruder may constitute a therapeutic intervention for persistent infections.

Pharmacologic interventions

The interest in understanding the interaction between xenobiotics and the microbiome is at least two-fold. On the one hand, pharmacologic treatment, particularly some antibiotics, is often associated with symptoms of gastrointestinal dysfunction (diarrhoea and constipation). On the other hand, the possibility that drugs could be used to modify the microbiota or its metabolites constitutes an interesting area for the development of new therapeutic strategies (Rajpal et al., 2015; Rodrigues et al., 2017). Indeed, a number of xenobiotics including proton pump inhibitors and antipsychotics were found to have profound effects on the intestinal microbiome (Maier et al., 2018).

For obvious reasons, antibiotics have a strong effect on the intestinal microbiota (Imhann et al., 2017). Depending on their mechanism of action, they eradicate susceptible bacteria and facilitate the expansion of resistant bacteria or those that are able to inactivate these drugs (Maurice et al., 2013). The cell wall-targeting antibiotic vancomycin for example increased the abundance of the gram$^-$ γ-proteobacteria and vancomycin-resistant strains of enterococci and enterobacteria while anaerobic bacteria and particularly *Bacteroidetes* decreased (Rodrigues et al., 2017). In contrast, the broad spectrum antibiotic Clarithromycin increases gram$^+$ aerobic cocci and antibiotic-resistant strains whilst decreasing *Enterococci* (Jernberg et al., 2010). Overall, antibiotics thus decrease microbial diversity but do not, even when combinations of different antibiotics are used, eradicate all gastrointestinal bacteria which is associated with a higher susceptibility to infection with pathogenic bacteria (Morgun et al., 2015; Ng et al., 2013; Rajpal et al., 2015).

Other xenobiotics have also been shown to modulate the microbiome (Maier et al., 2018; Zhernakova et al., 2016). Proton pump inhibitors (PPI) for example are associated with a higher risk of infection with pathogens such as *Campylobacter*, *Salmonella* and *Clostridium* and with higher abundances of

Streptococcaceae, *Enterococcaceae* and *Staphylococcocaceae*. In contrast, *Bifidobacteriaceae* and *Ruminococcaceae* are negatively affected by PPI (Imhann et al., 2017). These changes can be attributed to the PPI-induced suppression of gastric acid secretion which strongly inhibits growth of bacteria and direct effects of PPI on microbial gene expression but a direct effect of PPI on commensal bacteria has also been suggested (Freedberg et al., 2015; Maier et al., 2018).

Stress

Evidence for an effect of psychological stress on microbiota composition has emerged from dietary intervention studies with pro- and prebiotics that were shown to reduce stress-related parameters as well as salivary cortisol awakening response (Kato-kataoka et al., 2016; Schmidt et al., 2015). Since then, the effect of adult, post- and prenatal stress on the microbiome has been addressed in rodent models. For example, Pusceddu et al. report an increased abundance of *Akkermansia*, *Prevotella* and *Flexibacter* in female maternally separated rats (Pusceddu et al., 2015). In contrast, Moussaoui et al. report a decreased abundance of *Oscillospira*, *Ruminococcus*, *Lachnospira*, *Roseburia*, *Coprococcus* and *Akkermansia* and increases of *Enterococcus*, *Proteus* and *Turicibacter* in a rat model of limited nesting stress followed by brief maternal separation (Moussaoui et al., 2017). Socially isolated adult mice differed from control animals based on *Clostridium* (increase) and *Lactobacillus* (decrease) (Bailey et al., 2011; Golubeva et al., 2015). The microbiota of mice stressed by restraint or food and water deprivation was also characterised by an increase of *Lachnospiraceae* (belonging to the class of Clostridia) but more importantly a decrease of *Tannerella* and *PrevotellIa* (Bailey et al., 2010). Overall, the microbiome of stressed animals differed from control animals on a family and genus level but the changes appear to be stressor-related and alterations cannot be generalised across studies.

Although these studies clearly demonstrate that stress induces alterations of the microbiome, there is still a lack of understanding with regard to the underlying mechanisms (Lomax et al., 2010; O'Mahony et al., 2011). Stress increases the permeability of the intestine potentially via Corticotrophin-releasing hormone (CRF) which can lead to bacterial translocation past the intestinal epithelium (Foster et al., 2017; De Punder and Pruimboom, 2015). This induces an immune response (section 1.1.2) and thus, the secretion of antimicrobial peptides and antibodies from the intestinal epithelium which could, in turn, affect the microbiota. Stress also activates the sympathetic nervous system and this could either directly through norepinephrine receptor-like structures expressed by bacteria or through regulation of the digestive activity and nutrient availability alter microbiome composition (Rooks et al., 2017).

Animal species and strains

Based on the enormous variability of the human and mouse microbiome, it was suggested that species genetics have a strong influence on microbiome composition (Nguyen et al., 2015). Indeed, it was found that the abundance of *Prevotella* and *Akkermansia* is very low in mice compared to humans (Doherty et al., 2017) whereas mouse microbiomes are rich in *Lactobacillus* and *Turicibacter* (Firmicutes) as well as *Tannarella* and *Alistipes* (Bacteroides). In addition, species belonging to the phylum Tenericutes (*Mollicutes*) are more frequently associated with mice (Scott et al., 2017). In a seminal study however, where Muegge et al. (2011) compared the microbiome composition of 33 mammalian species with regard to their function, it was found that there was a low association of bacterial lineages or gene content with the phylogenetic relation of species. Instead, the high β-diversity of bacteria and their function was related to the diet of a particular species and it was concluded that dietary intake has a stronger influence on the functions of the core microbiome than species' genetics (Muegge et al., 2011) and this suggests that, in future studies, a better correlation between human and mouse studies might be achieved by changing the animals housing conditions and diet.

However, although dietary habits appear to be an overwhelming factor with regard to variability on a population level, the effect of genotype on the microbiome is a concern particularly in animal research using standardised diets. When exposed to a number of different diets, the microbiome of various mouse strains changed in agreement with Muegge et al. (2011). Yet, an increase of *Akkermansia*, *Lachnospiraceae* and *Clostridia* in response to a high fat/high sugar diet was observed in most investigated strains but not in 129S1/SvlmJ and NZO/HILtJ mice (Carmody et al., 2015; Holmes et al., 2017). Furthermore, conventional but not germfree mice C57Bl/6 mice become obese when they are fed various diets (Bäckhed et al., 2004a, 2007) whereas a higher susceptibility of conventional compared to germfree C3H mice to obesity was only observed when both were fed a Western diet (Fleissner et al., 2010). Additionally, colonisation of germfree mice with the microbiota from Balb/c or NIH Swiss mice results in alterations of behaviour specific to the strain (Bercik et al., 2011). From these studies, it can be concluded that attention should be paid when choosing a suitable mouse model and that confounding factors influencing the microbiome composition need to be considered.

Modulation by the human host

From what has been described so far, it appears that the composition of the microbiome is mostly affected by extrinsic factors and is not the target of specific regulation by the host. Given the wide-ranging benefits of some bacteria in contrast to others, it would be surprising if the host hadn't developed mechanisms to modify the composition of its microbiome. Consistent with this idea, Liu et

al. (2016) have shown that epithelial cells produce microRNAs that regulate the growth of commensal bacteria (Liu et al., 2016). Others have also identified microRNAs that specifically inhibit the growth and virulence of pathogenic bacteria (Duval et al., 2017). The stimuli that induce microRNA production still remain to be uncovered but certainly, microRNA represent an interesting target for future attempts to modulate the microbiome.

One aim of this section was to highlight the various factors that contribute to the variability of microbiome composition. Those are strongly interdependent and disentangling them will remain a challenge for the future. Contradictory findings are thought to be related to an insufficient sequencing sensitivity and technological effects of sampling and data analysis (Faith et al., 2013). The storage and processing of samples for example have been shown to influence the abundance of certain species. Therefore, standardisation will become particularly important to compare studies and draw meaningful conclusions.

1.1.2 The microbiome and the GI tract

The gastrointestinal tract has a surface area of 32 m^2 and is the body's largest contact point with the outside world (Helander and Fändriks, 2014). Colonisation with commensal bacteria has been appreciated for many years but we are only now starting to better understand the details of this symbiosis. In the following section, the close functional relationship between anatomical structures of the GI tract and commensal microbes will be described (Fig 2). A detailed description of the sensory systems will be the focus of section 2 (p 38).

Colonisation resistance

The mere presence of the gut microbiota in the intestinal lumen antagonises colonisation with pathogenic bacteria. Commensal bacteria occupy potential binding sites for pathogenic bacteria on host cells. Those include membrane-bound receptors and glycolipids expressed on the surface of epithelial cells. The intake of oral broad-spectrum antibiotics partially reduces the numbers of adherent commensal bacteria and increases the susceptibility to drug-resistant pathogenic bacteria (Morgun et al., 2015, Walker et al., 2013).

Commensal bacteria influence the availability of nutrients which may contribute to a reduced survival and growth of pathogenic bacteria. However, it is especially pathogenic bacteria that are metabolically

flexible and can derive energy from various sources. In fact, pathogens such as *Salmonella typhimurium* and *Clostridium difficile* use mucus-derived substances for their metabolism and thus, depend on the presence of mucus-degrading commensals that provide fucose and sialic acid for example. Similarly, mono- and disaccharides which are the preferred metabolite for pathogenic *Escherichia coli*, are more abundant in the presence of saccharolytic commensal bacteria (Pacheco et al., 2012). Thus, although competition for nutrients restricts the growth of some bacteria, it is not a strong selecting factor against pathogens (Baümler and Sperandio, 2016; Vogt et al., 2015).

In contrast, the ability of commensal bacteria to produce antimicrobial substances and inhibitors of bacterial proliferation may more specifically target pathogenic bacteria (Sánchez et al., 2010). Substances with known antimicrobial function have been found in the genomes of *Lactobacillus*, *Escherichia coli* and *Bifidobacterium* species and those can kill or inhibit the growth of *Klebsiella pneumonia*, *Yersinia pseudotuberculosis*, *Staphylococcus aureus* and *Salmonella typhimurium* in-vitro (Collins et al., 2017). In gram$^+$ bacteria, these constitute pore-forming peptides that are related to the antimicrobial cathelicidins and β-defensins produced by the host.

In addition to antimicrobial substances, gram$^+$ and gram$^-$ bacteria in the gut also secrete molecules that signal high population density (quorum sensing). These mediators constitute peptides (gram$^+$) and small molecules (gram$^-$) which have profound effects on bacterial gene expression. It has been shown that quorum sensing molecules antagonise QS from other, even closely related species (Canovas et al., 2016; Paharik et al., 2017; Qazi et al., 2006; Vasquez et al., 2017). However, some bacteria are able to "cheat" and activate QS-regulated genes without producing the respective QS molecule or respond to QS molecules from other species (Roy et al., 2010). *Bacteroides thetaiotaomicron* has also been shown to decrease expression of *E. coli* shiga toxin by secreting small molecules that are not involved in QS (De Sablet et al., 2009). This evidence for cross-talk of bacterial communities is highly relevant in the context of the intestine and further studies should investigate the effect of these interactions on microbial gene expression.

Epithelial barrier

It is a major function of the GI tract to protect the host from harmful stimuli present in the intestinal lumen. The epithelium which comprises absorptive enterocytes as well as specialised cells that produce mucus (Goblet cells), antimicrobial peptides (Paneth cells) and gut hormones (enteroendocrine cells), is an essential component of the intestinal barrier (Baümler and Sperandio, 2016; Kamada et al., 2013;

Pickard et al., 2017). In the following, the roles of these cells and their modulation by commensal/probiotic bacteria will be described.

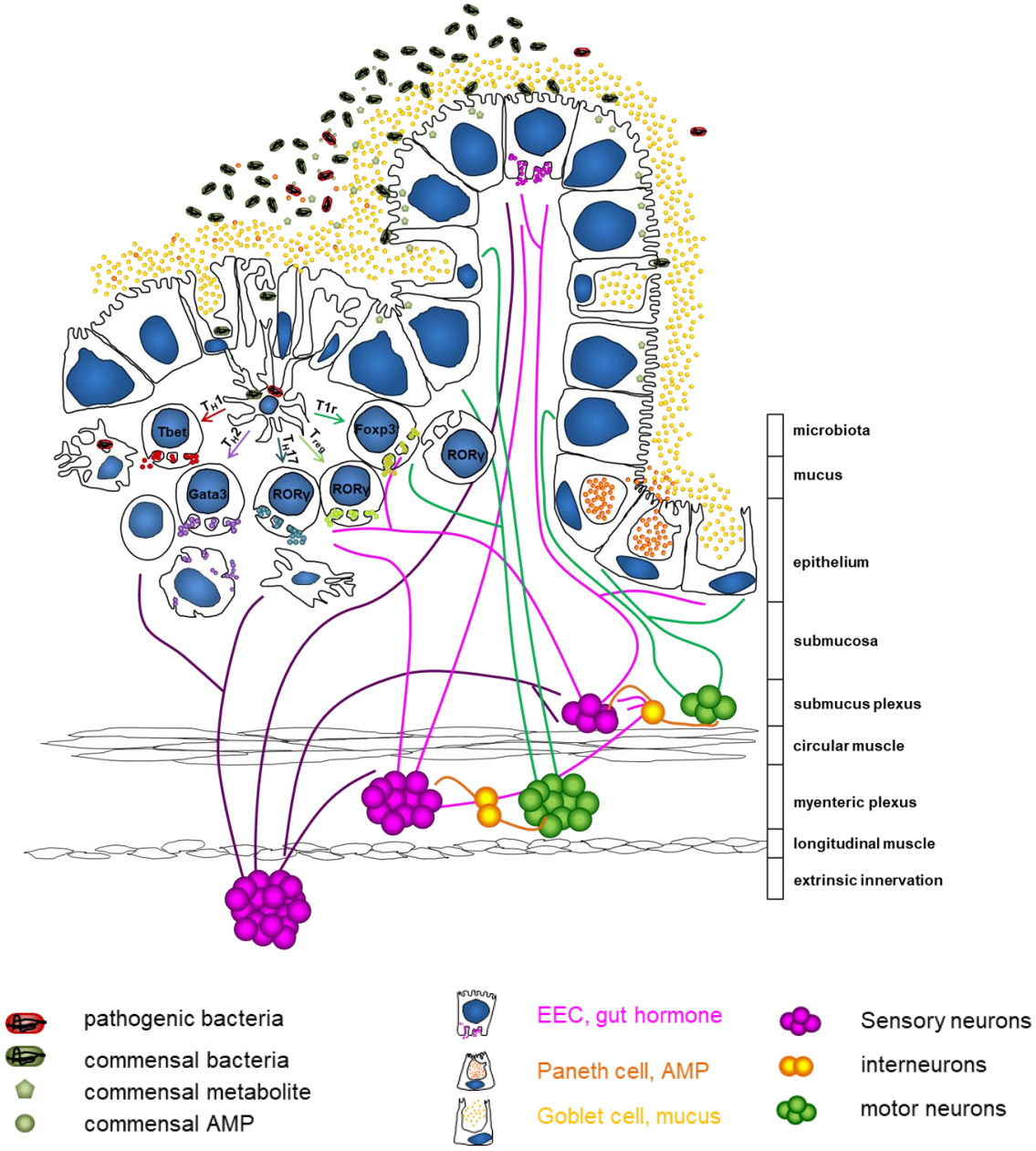


Figure 2: Intestinal microbes are in close contact with anatomical structures of the GI tract and affect their function. Commensal bacteria contribute to colonisation resistance, metabolism and the development and function of the immune and nervous system though their presence in the intestinal lumen, production of antimicrobial substances (AMP) and metabolites, their interaction with specialised epithelial cells (EEC, pink granules; Paneth cell, orange granules; Goblet cell, yellow granules), resident immune cells and neurons. Refer to the text for further details. Figure adapted from (Yoo and Mazmanian, 2017)

The integrity of the epithelial barrier is dependent on the expression of cell-cell and cell-matrix proteins. Tight-junction and adherence junction proteins are composed of claudins and occludins for example and it has been shown that commensal bacteria regulate their expression and consequently, epithelial permeability (OHland and MacNaughton, 2010). Incubation of epithelial cell lines with probiotic bacteria increases the expression of tight junction proteins. When epithelial cells or intestinal tissue are incubated with commensal bacteria, trans-epithelial resistance increases while FITC dextran passage decreases which is in line with a lower level of tissue permeability and ion movement (Jensen et al., 2012; Karimi et al., 2018). Furthermore, colonisation of germfree animals with commensal bacteria also decreases the amounts of fluorescein isothiocyanate (FITC) that translocate from the lumen into the tissue after oral gavage of the dye. Different bacterial strains differ in their protective capacity indicating that it is important which bacteria interact with epithelial cells (Sultana et al., 2013).

The interaction of epithelia and bacteria also modulates the expression of mucins and other defence proteins from specialised cells in the intestinal epithelium. Goblet cells are located in the crypts of the epithelium and produce mucins. These are composed of a protein moiety which is heavily glycosylated and thus, forms a gel-like protective layer covering the epithelium (Liévin-Le Moal and Servin, 2006). *Lactobacillus* species potently induce mucin secretion in colonic cell line via the production of a yet unidentified heat-resistant non-proteinaceous soluble mediator (Caballero-Franco et al., 2007) and *Bifidobacterium longum* have been shown to increase the thickness of the mucin layer in-vivo (Schroeder et al., 2017). Regulation of mucin secretion may constitute an advantage for commensals because, some of these can use mucins, particularly their sugar moieties, for energy metabolism.
With regard to Paneth cells, it is still a matter of debate whether commensal bacteria induce secretion from these immune-like cells. On the one hand, regenerating islet-derived 3-γ (RegIIIγ) and IgA production are markedly reduced in germfree animals and α-defensin secretion can be induced in-vitro by exposure of small intestinal crypts or cell lines to bacteria or bacterial products (Ayabe et al., 2000, 2002; Cash et al., 2006; Hapfelmeier et al., 2010). On the other hand, germfree mice also express defensin genes and are not devoid of Paneth cells indicating that commensal bacteria are not required for defensin expression or differentiation (Putsep et al., 2000). Thus, given the potential negative effects of Paneth cell mediators for bacteria including commensals, the microbiota might not contribute to a constitutive expression of these proteins but rather for regulating their turnover (Salzman et al., 2007).

Metabolism

The intestine also is essential for nutrient absorption and energy supply. Nutrients are taken up on the apical surface of enterocytes via passive or active transport. On the basolateral side, they are secreted into the interstitium and enter the circulation via the portal vein or lymphatics. The intestinal microbiota impacts the availability and composition of the absorbable molecules through metabolising ingested food components and synthesising essential (micro-) nutrients (Rowland et al., 2017).

The metabolism of commensal bacteria is adjusted to the available energy sources at different sites of the GI tract. In the upper gut, these include carbohydrates, amino acids and other metabolites that are generated by human digestive enzymes. In the lower gut, it is mostly indigestible food components that are available and because of the low oxygen concentration, most bacteria ferment these substrates. Based on their preferred substrates, bacteria can be categorised as being proteolytic or saccharolytic. The former express enzymes that enable the degradation of proteins and amino acids such as ureases and amino acid decarboxylases, transaminases and lyases (Muegge et al., 2011). Saccharolytic bacteria on the contrary metabolise dietary fibre (arabinoxylan, cellulose, β-glucan, fructan, resistant starch and lignin) which constitute a major component of plant products (Maukonen and Saarela, 2015; Scott et al., 2008; Sonnenburg et al., 2010), shorter fermentable carbohydrates (FOS, GOS, FODMAP) and host glycans. *Bifidobacteria* and *Lactobacillus* species are well-known for their potential to degrade FOS and GOS as well as mannans, sialylated glycans, galactose or fucosyloligosaccharides. Some species of the *Bacteroides* family have been shown to grow on porcine mucin *O*-glycans (Pudlo et al., 2015).
The end products of protein and carbohydrate fermentation include short chain fatty acids (SCFA). Almost 20 % of the species from different phyla (Vital et al., 2014) and particularly saccharolytic bacteria produce butyrate and propionate which constitute important bacteria-host signalling molecules. Colonocytes use butyrate for their energy metabolism and it also contributes to immune cell differentiation as well as hormone secretion from enteroendocrine cells. In contrast, proteolytic bacteria preferentially produce valerate and iso-valerate via deamination or decarboxylation of dietary or host proteins. These cannot be absorbed and by-products of proteolysis such as amines, indoles, hydrogen sulfide and branched chain fatty acids are known to have negative effects on human health (Rowland et al., 2017). Consequently, the metabolite and SCFA profile in particular can be used as an indicator of microbiome composition but is not linked to the presence or absence of specific bacteria as some strains can alternate between fermentation pathways when different substrates are provided.

Members of the microbiota also synthesise nutrients that are essential for the host. Many species of the *Bacteroidetes* phylum (90 %) but also smaller proportions of *Fusobacteria*, *Proteobacteria* and *Actinobacteria* have the potential to synthesise different B vitamins including thiamine (B1), folate, biotin (B7) and cobalamin (B12) (Rowland et al., 2017; Yatsunenko et al., 2012). In addition, gene transcripts for the enzymes catalysing glutamate synthesis have been found in microbiome analyses particularly in individuals and animals on a low protein diet (David et al., 2014a).

Lastly, the microbiota is also involved in de-conjugating and metabolising bile acids which is important for their re-uptake into the enterohepatic recirculation and plays a role in xenobiotics inactivation (Golubeva et al., 2017; Rowland et al., 2017).

Development of the Immune response

The intestinal tract is a large immune organ and constitutes about 70-80 % of the body's immune cells. Resident immune cells include dendritic cells, macrophages, granulocytes (neutrophils, eosinophils and basophils), mast cells and many types of lymphocytes (Thaiss et al., 2016). While some diffuse dendritic cells and macrophages can be found throughout the gastrointestinal wall, most immune cells are concentrated in specialised lymphoid structures such as Peyer's Patches and lymph follicles. Luminal and mucosal antigens are sampled by epithelial M cells and dendritic cells and this induces the differentiation of lymphocytes.

The gut microbiota plays an important role in the establishment an immune environment that tolerates commensal bacteria whilst effectively combating pathogens. Hyper-responsiveness to commensal antigens would be detrimental given their beneficial effects and high abundance. It has been shown that commensal/probiotic *Lactobacillus* and *Bifidobacterium* species modulate mucosal cytokine levels (decrease TNF-α,, IFN-γ for example) which impacts the activity of resident immune cells (Kotzamanidis et al., 2010; Llewellyn and Foey, 2017). Additionally, pre-incubation of neutrophils with microbiota-derived peptidoglycan enhances their antibacterial activity against pathogens (Clarke et al., 2010). These studies exemplify a conditioning effect of the microbiome on the innate immune system (Khosravi et al., 2014; Round and Mazmanian, 2009). In addition, the impact of the microbiota on the adaptive immune response has been investigated and those studies implicated the transcription factor retinoic acid receptor (RAR)-related orphan-like (ROR)-γ in the sensing of commensal bacteria.
ROR-γ is initially expressed by most lymphocytes in intestinal lymph follicles. During differentiation, only some subpopulations maintain a high expression of ROR-γ and those have been shown to be

particularly responsive to signals from the intestinal microbiota. A major cell type that expresses high levels of ROR-γ are the T_H17 ($CD4^+$) cells (Ivanov et al., 2008). They are induced by segmented filamentous bacteria (SFB) from the cytophaga-flavobacter-bacteroides group but not by *Enterococcus faecalis* or the altered Schaedler's flora (Farkas et al., 2015; Ivanov et al., 2009) and secrete IL-17 which stimulates recruitment and phagocytic activity of neutrophils. T_H17 cells are associated with innate lymphoid cells ILC3 which also express ROR-γ. These appear to have an inhibitory functions on T_H17 activity because ablation of these cells induces low grade inflammation (Ohnmacht, 2016). The second major ROR-γ^+ T cell type constitutes a subpopulation of regulatory T cells. These cells acquire expression of the T_{reg} marker FOXP3 and are particularly prominent in the small and large intestine (Ai et al., 2014). It has been found that the most prominent ROR-γ-T_{reg} cell-inducing bacteria include members of the *Clostridia*, *Bacillus fragilis*, *Bacteroides thetaiotaomicron*, *Staphylococcus saprophyticus* and *Clostridium rhamnosum* which also metabolise carbohydrates to SCFA (Atarashi et al., 2011; Round and Mazmanian, 2010; Sefik et al., 2015). Those $FOXP3^+$ cells produce either IL-10 or TGF-β which inhibit T_H17 and T_H1 differentiation. Another T_{reg} subpopulation (T1r) which does not express FOXP3 ($Foxp3^-$), can be induced by *Bifidobacterium* and also secretes the anti-inflammatory IL-10 (Jeon et al., 2012). Lastly, ROR-γ is expressed by population of T cells which is similar to natural killer cells (Sanos et al., 2009). Their abundance increases upon colonisation of germfree animals and subsequent IL-22 release contributes to the induction of antimicrobial peptides (AMP) in the intestinal epithelium (Sanos et al., 2009).

In contrast to the T cell lineages described so far, it is not clear yet whether the two originally described T_H1 and T_H2 subpopulations also respond to commensal bacteria. Alternatively, these would be more implicated in the inflammatory response to pathogenic microbes and require microbial as well as signals of tissue destruction for their activation. Bacteria induce a T_H1 polarisation via T-bet activation whereas extracellular antigens and helminths induce the Gata3 transcription factor leading to a T_H2 response. The main cytokines, IFN-γ and IL-5, activate macrophages and eosinophils, mast cells and B cell differentiation respectively (Powell and MacDonald, 2017).

Neuronal Development and function

Neurons are abundantly present in the gastrointestinal tract (section 2, p 38). They comprise more than 100 million cells belonging to the intrinsic enteric nervous system and additional 100.000 extrinsic neurons (Yoo and Mazmanian, 2017). As shown in Figure 2 (p 9), intrinsic enteric neurons are located in organised neuronal networks underneath the mucosa (submucous plexus) and in between the muscle layers (myenteric plexus). Extrinsic neurons are located in ganglia outside the intestinal wall.

Studies using germfree mice and probiotic interventions have established an important function of the microbiota for neuronal development and function. *Lactobacillus* species were found to alter neuronal activity within the enteric nervous system (Wang et al., 2009, 2010; Wu et al., 2013) resulting in changes of GI motility. The lack of a microbiota results in profound behavioural as well as biochemical alterations in the CNS. Germfree mice display a reduced social motivation, novelty object exploration and an altered anxiety behaviour as well as an increased restraint stress-induced Adrenocorticotropic hormone (ACTH) and corticosterone response, serotonin expression and a decreased Brain-derived neurotrophic factor (BNDF) expression (Clarke et al., 2013; Desbonnet et al., 2014; Heijtz et al., 2011; Neufeld et al., 2011; Sudo et al., 2004). Some of these changes can be reversed by colonisation with a complete microbiota or non-pathogenic commensal strains early in life which indicates that these bacteria contribute to the alterations of CNS function. These effects have been shown to depend on gut-brain communication (vagus nerve) as well as expression of peptidoglycan-sensing molecule (Pglyrp2) in sensory neurons (Arentsen et al., 2017; Bravo et al., 2011). In the latter study, Arentsen et al. (2017) recapitulated the germfree phenotype via knock out of the bacterial sensing receptor Pglyrp2. As will be described later (section 1.1.5, section 3), neurons of the peripheral and enteric nervous system also express other receptors for bacterial products.
These studies indicated that neuronal bacteria sensing contributed to the observed behavioural and neurochemical changes but bacteria-induced release of gut hormones or the effects of the microbiota on immune function may also be involved (Lach et al., 2018).

This section aimed to provide an overview on the anatomy of the gastrointestinal tract and how commensal bacteria contribute to physiology. It is important to note that although commensal bacteria share many genes and may be functionally redundant (Kurokawa et al., 2007), specific interactions do exist as well and it will be interesting to identify these.

1.1.3 Detection of commensal bacteria

To exert their multiple effects on physiological functions, bacteria needs to interact with the host. This is mediated by the expression of ligands for host receptors. Traditionally, it was thought that it is the immune system that recognises bacteria but it is now well-established that other cells also express receptors for bacterial sensing.

Receptor-mediated bacterial sensing

Commensal bacteria produce a wide range of different substances that can be detected by host cells. Given the growing number of host proteins involved in bacteria sensing, it appears that being able to detect bacteria and their compounds is an evolutionary advantage for the host.

Pattern recognition receptors (PRR) were among the first receptors that were shown to be involved in bacterial recognition (Blander and Sander, 2012; Mogensen, 2009). While they were initially thought to be particularly important for the detection of pathogen associated molecular patterns (PAMP), we now know that the PAMPs detected by PRRs only allow a broad classification of microorganisms and that additional signals are necessary to distinguish commensal and pathogenic intruders (see Blander et al. (2012), Mogensen (2009) and section 1.2).

Classical PRRs include Toll-like receptors (TLR), nucleotide-binding domain and leucine-rich repeat containing proteins (NOD), NOD-like receptors (NLR), AIM (absent in melanoma)-like receptors (ALR), C-type lectins (CLR) and retinoic acid inducible gene I (RIGI)-like receptors (RLR). The agonists of these receptors are summarised in table 1 (p 30) and include wide range of chemically different substances produced by bacteria, viruses and fungi (Neish, 2009).

(1) Toll-like receptors are the best known membrane-bound receptors of the innate immune system. They detect mostly structural components of the invading helmith, fungi, bacteria or virus. Activation of TLRs induces the recruitment of MyD88 and IL-1 receptor-associated kinase-4 (IRAK-4) which ultimately leads to activation of nF-κB via inactivation of its inhibitor IKK. Stress signalling is also induced via activation of Janus kinase (JNK) and mitogen-activated protein kinase (MAPK) p38 but other MyD88- and nF-κB-independent signalling pathways exist as well. Overall, TLR signalling is particularly involved in regulating cellular survival (anti-apoptotic) and the transcription of pro-inflammatory cytokines in immune cells (Li et al., 2010).

(2) NOD receptors on the contrary recognise invading intracellular bacteria and similar to TLR activate nF-κB (Chaput et al., 2013).

(3) The cytosolic NLR and ALR receptor families comprise 23 (34) and 4 (13) members in human (mice), respectively and are activated by a broad range of microbial substances. They are part of multi-protein inflammasome complexes which also comprise the adaptor protein ASC[2] and the inactive procaspase-1 zymogen. Upon ligand binding to its NLR or ALR, inflammasome activation induces cleavage and activation of procaspase-1. Caspase-1 cleaves the IL-1β precursor and induces caspase-dependent apoptosis (Chaput et al., 2013; Storek and Monack, 2015).

[2] apoptosis-associated speck-like protein containing a CARD domain (ASC)

More recently, it has emerged that other receptors particularly members of the G protein-coupled receptor (GPCR) family can also be activated by various bacterial compounds and particularly their metabolites (Husted et al., 2017). GPCRs constitute a major part of the human genome. To date, as many as 800 GPCRs have been identified based on the predicted seven transmembrane domain (7TM) receptor structure and they are particularly involved in sensation as well as intercellular communication. While their extracellular N-terminus is responsible for ligand activation, the intracellular domain is important for the signal transmission via coupling to heterotrimeric G proteins. The α-subunit either inhibits ($G_{\alpha i}$) or activates ($G_{\alpha s}$) adenylyl cyclate (AC) or phospholipase C (PLC)-β ($G_{\alpha q}$) whereas the βγ-subunits can activate phosphoinoside-3-kinase (PI3K) and also act as modulators of ion channels such as GIRKs[3] and voltage-gated Ca^{2+} channels (Alexander et al., 2017). Those are summarised in table 1 and will be briefly described below.

(1) Many GPCRs detect metabolites which can derive from commensal bacteria in the gut but are also produced by humans and therefore, expression of these receptors does not only function to detect bacteria. These include SCFA receptors (GPR41, GPR43), lactate and succinate receptors (GPR91, GPR91). In contrast, FPRs, Taars and T2R might be more specifically involved in bacterial sensing.

(2) Bacteria also produce substances that mimic human molecules and thus, these can activate GPCRs that are involved in neurotransmission (GABA, PTGER) and immune defence (S1PR4).

Two other non-GPCR have also been described to be involved in bacterial sensing. The ligand and temperature-gated ion channel TRPA1 has recently been shown to be activated by lipopolysaccharide (LPS) independent of TLR4 which suggests that other TRP channels might also be able to respond to bacterial products (Meseguer et al., 2014). In addition, the cytosolic aryl hydrocarbon receptors (AHRs) was found to be involved in the regulation of metabolism by commensal bacteria (Korecka et al., 2016).

This shows that a number of receptors not traditionally involved in bacterial sensing might actually have evolved to signal the presence of intruders. It is tempting to speculate that among the many orphan receptors in the human genome, at least some will also respond to bacterial products.

[3] G-protein-regulated inwardly rectifying K+ channels (GIRKs)

Table 1: Overview of receptors involved in bacterial sensing. These include "traditional" PRRs and receptors for metabolites released from bacteria. Summarised from (Chaput et al., 2013; Cohen et al., 2017; Husted et al., 2017; Neish, 2009), PGN, peptidoglycan; SCFA, short chain fatty acids; Trp, tryptophan

Receptor		Ligand
TLR1	Toll-like receptor	Lipopeptide
TLR2		Lipoteichoic acid
TLR3		dsRNA
TLR4		Lipopolysaccharide
TLR5		Flagellin
TLR6		Lipoprotein, LTA
TLR7		viral RNA
TLR8		viral RNA
TLR9		CpG DNA
TLR11		Profilin
NOD1	nucleotide-binding domain, leucine rich repeat containing protein receptor	gram$^-$ PGN
NOD2		PGN
MHCI	major histocompatibility complex	T cell receptor
MHCII		
NLR	NOD-like receptor (NLRC, NLRP, NLRX)	Intracellular microbial antigens
RLR	retinoic acid inducible gene I (RIGI)-like receptors	viral RNA
CLR	C-type lectin receptor	fungal carbohydrates
ALR	AIM (absent in melanoma) like receptor	dsDNA

Receptor		Ligand
GPR41	G protein coupled receptor	SCFA
GPR43		SCFA
GPR35		Trp metabolites
GPR84		MCFA, capric acid
GPR119		Acetyl-amines/glycerols
GPR132		oxidised FA
GPBAR		bile acids
FPR	Formyl-peptide receptor	formylated peptides
GPR81		Lactate
GPR91		succhinate
GABAA	γ-Amino buturic acid receptor	GABA
GABAB		
PTGER	Prostaglandin	PGE, bacterial product
T2R	Bitter taste receptor	acyl homoserin lacton
Taar	Trace amine-associated	Tyramine
TRPV1	Transient receptor potential vanilloid 1	lipopolysaccharide
S1PR4	lysosphingolipid sphingosine 1P receptor	Sphingosine metabolite, bacterial product
AHR	Aryl hydrocarbon Receptor	pollutants, kynurenine and planar indoles (Trp)

Non-immune cells involved in bacterial sensing

The large variety of receptors that are involved in bacterial sensing indicates that it is important to be able to detect and respond to bacteria and their metabolites. It is therefore not surprising that cells other than immune cells also express some of these receptors.

In the GIT, Gourbeyre et al. (2015) described the distribution of PRRs in different areas and along the crypt villus axis. Consistent with being primarily involved in immune cell bacterial sensing, they found that most TLRs are highly expressed in mesenteric lymph nodes and peyer's patches and less abundant in the duodenum compared to jejenum and ileum. TLR3 and TLR5 as well as RIG-I were found in vili whereas crypts did only express low levels of PRRs (Gourbeyre et al., 2015). With regard to the cell types that are involved in bacterial sensing, it is particularly secretory cells (enteroendocrine cells, Paneth cells and Goblet cells) that are involved in bacterial sensing. They release their mediators in response to bacteria which may initiate host defence mechanisms and sensation (Lach et al., 2018; Naafs, 2018).

It has also been described that neurons can respond to bacterial products (Chiu et al., 2013; Mao et al., 2013; Meseguer et al., 2014; Ochoa-Cortes et al., 2010) and that this results in changes of neuronal activity (section 3, p 52) but whether and how bacteria interact with neurons in the intestinal tract has remained largely un-investigated.

1.2 Infection and pathogens

In sharp contrast to the beneficial effects of colonisation with commensal bacteria (section 1.1), pathogenic bacteria cause acute and chronic illness. Bacterial infection has long been a major cause for human death but since the discovery of antibiotics, the lethality of bacterial infections has decreased. Gut infections are most frequently caused by bacteria present in food but transmission of pathogenic bacteria also occurs via contaminated surfaces or contact with other people (Biomerieux-diagnostics; Scallan et al., 2011). They are associated with acute reflex behaviours such as vomiting, emesis and diarrhoea as well as behavioural changes (nausea, anorexia, social isolation, discomfort) and fever. Some patients also experience episodes of GI pain. Because infective gastroenteritis is normally self-limiting, medical interventions are often not required and the trigger remains unidentified (Graves, 2013; Kitamoto et al., 2016). Particularly in vulnerable populations (children, elderly, hospital) and in low/middle income countries however, gastroenteritis remains a serious health concern (Bump et al., 2013) and it is also appreciated as a risk factors for the development of a number of chronic inflammatory diseases (section 3.3) such as irritable bowel syndrome (Spiller, 2003), central nervous system (CNS) diseases etc. Thus, there is a need to understand what distinguishes these pathogenic from the resident bacteria in the gut.

1.2.1 Virulence factors

A common feature of pathogenic bacteria is the production of virulence factors/toxins. These are substances that can cause symptoms of an infection without the need of the bacteria itself and constitute a group of molecules that are diverse with regard to their chemical nature, mode of action and target structures. Endotoxins such as lipopolysaccharide and lipoteichoic acid are considered as virulence factors but because those constitute the cell wall of many also non-pathogenic gram$^-$ and gram$^+$ bacteria, they will not be further discussed here (refer to Table 1, p 30). In contrast, exotoxin production is mostly restricted to a limited number of bacteria and closely linked to the disease symptoms during bacterial infections (Connor et al., 2018; Kitamoto et al., 2016). Among them, toxins produced by

Staphylocci (Firmicutes), *Clostridia* (Firmicutes), *Salmonellae* (Proteobacteria) and *Escherichia* (Proteobacteria) are most relevant to human diseases (Table 2). The main mechanisms of action include receptor activation (type I), disruption of the cell membrane (type II) and intracellular activity (type III). These toxins interact with host cells via receptor proteins, glycoproteins or lipids that are present at the target cell membrane. Particularly type I and type III toxins display specificity for distinct cell types. For example, clostridial neurotoxins (type III) require the presence of a ganglioside for their activity. This is only expressed on sensory neurons and therefore, it is these cells that are targeted by Botulinum and Tetanus toxin. The requirement of receptors is also reported for many staphylococcal toxins and those will be described in section 1.2.3 (p 34).

Other virulence factors with different modes of action also exist. In particular, bacteria such as *S. aureus* produce proteases which degrade the extracellular matrix and components of the immune response which increases their ability to invade and persist in the host (Kitamoto et al., 2016).

Table 2: Different classes of soluble virulence factors are released by pathogenic bacteria. Summarised from (Peraro and van der Goot, 2016; Wilson and Salyers, 2011)
eta-etd, Exfoliative toxins; pvl, Panton-Valentine Leukocidin; tcdA, toxin *Clostridium difficile*

Toxin	Gene name	species
Type I toxin (receptor-mediated acitivity)		
- enterotoxins	sea-seo	*S. aureus*
- toxic-shock syndrome toxin	tst	*S. aureus*
Type II toxin (disruption of cell membrane)		
- leucodicins	pvl, lukD, lukE, lukF, lukM, lukS	*S. aureus*
- hemolysins	hla, hld, hlg, hly	*S. aureus*
- perfringolysin	pfo	*C. perfringens*
- phospholipases	plc, pld	*C. perfringens*
- Sphingomyelinase	hlb	*S. aureus*
- β-toxin	eta-etd	*S. aureus*
- colicins	caa, cea, cia, cna	*E. coli*
- cytolysin A	clyA	*S. enterica*
Type III toxin (membrane translocation)		
- botulinum toxins	botA-botF	*C. botulinum*
- tetanus toxin	tet	*C. tetani*
- heat-labile enterotoxin	elt, etx	*E. coli*
- glucosyltransferases	tcdA, tcdB	*C. difficile*
- cytolethal distending toxins	cdtA-cdtC	*E. coli*
- cytotoxic necrotising factors	cnf1-3	*E. coli*

1.2.2 Regulation of virulence factor production

Virulence factors are essential for the invasion, persistence and spread of bacteria during infections but their production also consumes substantial amounts of energy and resources. Therefore, it is important that bacteria are able to adjust virulence factor production to environmental conditions such as temperature, pH, osmolality as well as oxygen, ion, carbon and nitrogen availability (Burgui et al., 2018).

Similar to eukaryotes, bacteria are able to sense their environment. In gram$^+$ bacteria, the sensory machinery usually consists of two-component systems (TCS). These are composed of a membrane-bound sensor kinase that is activated by extracellular cues and catalyses the phosphorylation of an intracellular response regulator. The phosphorylated regulator affects target gene expression either directly through binding to their promotor or indirectly through expression of regulatory RNA (RNAIII). This pathway is exemplified for staphylococcal sensing of high population density (quorum sensing) shown in Figure 3. Quorum sensing (QS) is a particularly strong stimulus that causes profound changes of bacterial gene expression and increases the production of virulence factors (Chapman et al., 2017a; Le and Otto, 2015). Activators of QS in gram$^+$ bacteria are auto-inducing peptides that are secreted during bacterial growth. In addition to TCS, the regulation of transcriptional activity in gram$^+$ bacteria involve the modulation of RNA polymerase activity through sigma factors which also respond to environmental cues.

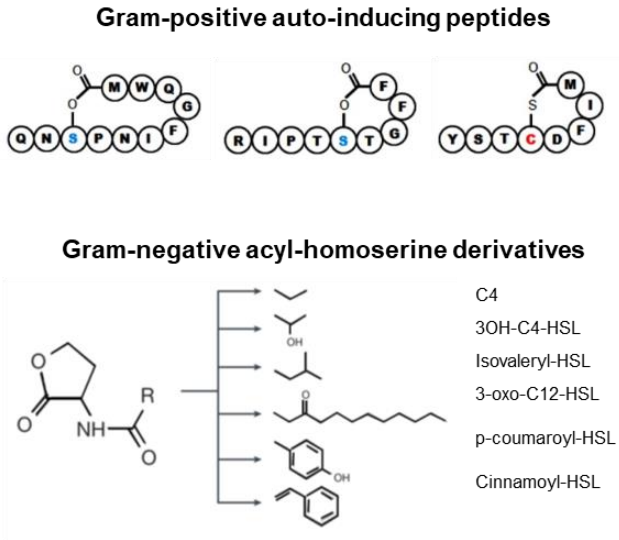


Figure 3: Quorum sensing in gram$^+$ bacteria depends on auto-inducing peptides. On the contrary, gram$^-$ bacteria produce small molecules that bind intracellular receptors. (Le and Otto, 2015; Sturme et al., 2002)

Gram$^-$ bacteria also respond to environmental signals such as quorum sensing. The molecules involved in gram$^-$ QS are chemically different from the gram$^+$ auto-inducing peptides. They constitute either acyl-homoserine lactones (AHL) or derivatives of S-adenosylmethionine that can cross the bacterial membrane and bind intracellular receptors (Papenfort and Bassler, 2016). As the QS molecules from gram$^+$ bacteria, AHLs are specific for different types of bacteria and regulate the expression of virulence factors. For example, the AHL released by *Pseudomonas aeruginosa* activate the LasI/LasR proteins and regulate the production of exoproteases. This indicates that QS molecules have important functions in many bacteria (Gobbetti et al., 2007).

1.2.3 Virulence factors during *S. aureus* infection

Among all disease-inducing bacteria, the opportunistic pathogen *Staphylococcus aureus* stands out for being able to produce large numbers of virulence factors and infecting a variety of different tissues whilst simultaneously being part of the healthy microbiota. Infection with (pathogenic) *S. aureus* but also other bacteria initiates a multistep process which involves the innate as well as the adaptive immune system and does not only aim to eradicate/killing the intruder but establishes inflammation memory (Hansen et al., 2011; Kumar et al., 2013).

General aspects of S. aureus infection

Because *S. aureus* is a gram$^+$ bacterium, the primary recognition mechanism through the innate immune system depends on peptidoglycan (PGN). PGN and lipoteichoic acid (LTA) interact with TLR2, TLR6 and NOD receptors of phagocytic cells which, via nF-κB signalling, induces the secretion of pro-inflammatory cytokines (Odendall and Kagan, 2017). In addition, opsonisation of the bacterial surface with complement factors or antibodies enables phagocytic uptake and and intracellular killing of the bacterium. Phagocytosis is also required for the processing and presentation of *S. aureus* antigens in major histocompatibility complexes (MHC). This, together with the cytokine environment, drives the differentiation of T lymphocytes (Gaspari et al., 2017; Krishna and Miller, 2012). During *S. aureus* infection, phagocytes release Interleukin-1β, -17 and -6 as well as interferon-γ which are important for T cell differentiation into a T_H1 or T_H17 phenotype (Ben-Sasson et al., 2009; Bröker et al., 2016; Ilarregui et al., 2016; Santarlasci et al., 2013). The T_H17 cytokines mediate the recruitment and activation of neutrophils. They increase the permeability of local blood vessels which facilitates neutrophil diapedesis and induce effective killing of phagocytosed *S. aureus* through myeloperoxidase and NADPH oxidase (Karauzum and Datta, 2017; van Kessel et al., 2014).

The success of *S. aureus* as an invasive pathogen is due to its production of virulence factors that enable it to escape the immune system. Pore-forming toxins such as α,-haemolysin (Hla), leucocidins (Hlg, LukAB, LukED, Pvl) and phenol-soluble modulins (Psm) efficiently lyse host immune cells whereas other virulence factors interfere with neutrophil-mediated killing, complement binding and phagocytosis, neutrophil extravasation or lymphocyte differentiation (Thammavongsa et al., 2015). These include a myriad of receptor antagonists (FLIPr-L, CHIPS, SSL[4]), immunoglobulin-binding proteins (Cna, SpA, Sbi[5]) and enzymes (Aur, AdsA, SodA, SodM, KatG, AhpC[6]). Overall, this impairs the successful eradication of pathogenic *S. aureus* and promotes its intracellular survival which is thought to constitute a source for chronic *S. aureus* infections (Fraunholz and Sinha, 2012).

S. aureus infects different sites of the body

It is important to note that some but not all strains of *S. aureus* carry genes for the above mentioned virulence factors. In fact, even closely related *S. aureus* strains produce different arrays of virulence factors and this partially explains why it is these strains that constitute a particular danger for human health. *S. aureus* is a leading cause of bacteraemia (blood infection) which can be associated with infections of skin and soft tissue (SSTI), heart (infective endocarditis, IE), catheters (line-related) and the respiratory tract (pneumonia). Invasion of these different tissues depends on cell-wall associated factors (clumping factors), pore-forming toxins (Pvl, Hla, Psm), microbial surface components recognizing adhesive matrix molecules (MSCRAMM) and Pvl/Hla for IE, SSTI, bone infection and pneumonia, respectively. This indicates that specific virulence factors are required and that it *S. aureus*'s diversity that contributes to the wide range of SA-induced diseases (Tong et al., 2015).

Prior colonisation is a known risk factor for *S. aureus* infection (Huang and Platt, 2003). Traditionally, nasal carriage is assessed but it is also reported that the intestine constitutes a potential reservoir for infection (Acton et al., 2009; Bhalla et al., 2007; Donskey, 2004). Intestinal *S. aureus* are frequently found in hospitalised patients but also in children, overweight pregnant women and IBS subjects (Collado et al., 2008; Lundell et al., 2007; Rinttilä et al., 2011). The bacteria could originate from other sites of the body particularly the nares which are colonised with *S. aureus* in about one third of the

[4] formyl peptide receptor-like 1 inhibitor (FLIPr), FLIPr-like (FLIPrL), chemotaxis inhibitory protein, staphylococcal superantigen-like (SSL)

[5] collagen adhesin (Cna), staphylococcal protein A (SpA) and staphylococcal binder of immunoglobulin (Sbi)

[6] Aureolysin (Aur), adenosine-synthesizing enzyme (AdsA), superoxide dismutase A (SodA, SodM, the catalase KatG, alkylhydroperoxide reductase (AhpC)

general population, the skin or the blood. Alternatively, contaminated food is also a possible source of SA. Staphylococcal food poisoning is particularly linked to the consumption of improperly handled and cooked food and symptoms include vomiting, nausea, stomach cramps and sometimes diarrhoea (Denayer et al., 2017). These symptoms can be induced by heat-stabile staphylococcal enterotoxins, particularly SEA, and leucocidin lukED (Gravet et al., 1999; Hu and Nakane, 2014) whereas cell wall-associated wall-teichoic acid (WTA) and clumping factor A (ClfA) are important for intestinal colonisation (Misawa et al., 2015).

Regulation and recognition of virulence factors

In section 1.2.2 (p 33), it has been described that virulence factor expression is regulated by environmental cues. *S. aureus* has 16 TCS (Burgui et al., 2018) and among them, at least 7 are important for virulence factor production in response to quorum sensing (AgrCA, SaeSR), acidic conditions (ChvGI), autolysis (ArlSR, LytRS), and oxygen (AlKR, SrrBA). QS regulation of virulence factor secretion has been most extensively studied because mutants of the accessory gene regulator (agr) have an alleviated capacity to induce severe diseases and thus, antagonists of agr could be used as treatment for staphylococcal diseases.

The Agr TCS of *S. aureus* consists of the sensor kinase AgrC and the transcription regulator AgrA (Figure 4). High population density increases the secretion of auto-inducing peptides (AIP). These are specific for different groups of *S. aureus* strains and activate the receptor histidine kinase AgrC. Via phosphorylation cascades, this signal leads to activation of the response regulator AgrA which binds to the promoter of immediate target genes and RNAIII. Thus, agrA directly increases expression of agr components themselves, metabolic enzymes, phenol soluble modulins and additional virulence factors via RNAIII-mediated stabilisation of the target gene's RNA. Haemolysins (α,-, β- and γ-haemolysin), leukocidins (Panton-Valentine leukocidin (Pvl), LukAB and LukED) and staphylococcal enterotoxins (sea, sea, seb, sec, sed, see) all fall into the latter category of indirectly regulated genes (Le and Otto, 2015).

As shown in Table 2 (p 32), many of the toxins produced by *S. aureus* affect target cells by disrupting the cell membrane. After their discovery, it was thought that this is a rather unselective process but it is now established that the disruption of the cell membrane by staphylococcal pore-forming toxins (PFT) requires the presence of proteinaceous and non-proteinaceous receptors (Seilie and Bubeck Wardenburg, 2017; Spaan et al., 2017; Vandenesch et al., 2012a).

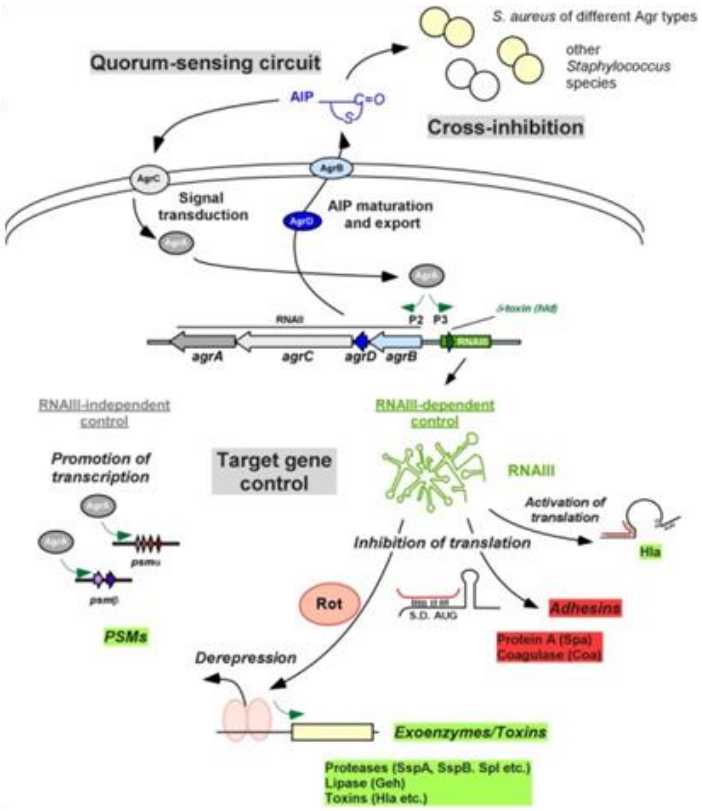


Figure 4: Quorum sensing pathways in *Staphylococcus aureus*. As other gram⁺ bacteria, It produces auto-inducing peptides which activate two-component systems (Le and Otto, 2015).

The lytic activities of the PFT α-haemolysin (α-toxin, Hla) are the most studied among the *S. aureus* toxins. It constitutes a 33 kDa soluble monomer that oligeromerises upon membrane binding and forms a heptameric channel that allows the passage of ions and nucleotides (Menestrina, 1986; Seilie and Bubeck Wardenburg, 2017). It has long been thought that Hla binding depends on a lipid sensor on the target cell membrane because depletion of liposomes from phosphatidylcholine, sphingomyelin or cholesterol reduces Hla's lytic activities (Valeva et al., 2006). At least since 2010 however, it is clear that a disintergrin and metalloproteinase domain (ADAM)-10 is required for Hla-mediated cytotoxicity and inflammasome activation (Ezekwe et al., 2016; Wilke and Wardenburg, 2010).

Proteinaceous receptors for other *S. aureus* leukocodins have also been described. Leukocidins (Pvl, LukAB, LukED, HlgAB, HlgCB), in contrast to Hla, consist of two different subunits which is why they are also referred to as bi-component leukocidins. All leukocidins, except for LukAB which dimerises shortly after secretion, combine and hetero-oligomerise after binding of the S component (LukS-PV,

LukE, HlgA, HlgC)[7] to the receptor at the host cell membrane. Most S components can bind to more than one receptor (Seilie and Bubeck Wardenburg, 2017) and there is also evidence that they can not only recruit cognate but also non-cognate F components (Spaan et al., 2017). Receptors involved in S component binding include the chemokine receptors CCR1, CCR2, CCR5, CXCR1 and CXCR2 as well as the complement factor receptor C5aR and CD11b (Alonzo III et al., 2012; Spaan et al., 2013). Another class of membrane-disrupting toxins produced by *S. aureus* has been identified lately (Wang et al., 2007). Phenol-soluble modulins constitute a group of short (20-40 aa) amphipathic peptides that bind liquid-disordered membrane domain because of their biochemical properties and cytotoxic at high concentrations. At sublytic concentrations, they can bind and activate formyl-peptide receptor-2 (FPR2) but in contrast to PFTs, this is not required for Psm-mediated cytotoxicity (Armbruster et al., 2016a; Kretschmer et al., 2010).

Most of the receptors involved in PFT binding belong to the family of G protein-coupled receptors and thus, interaction of PFT does not only enable pore formation but also modulates intracellular signalling pathways which also contribute to disease pathogenesis (Vandenesch et al., 2012a).

2 Innervation of the intestinal tract

In addition to its essential role in the digestive processes and nutrient absorption, the GI tract also constitutes a sensory organ. Two populations of sensory neurons are present in the GI tract and those are involved in initiating enteric (peristalsis and secretion), mesenteric (for example gastric emptying) and spinal (pain) reflexes (Mayer, 2011).

2.1 Extrinsic innervation

The extrinsic sensory innervation of the GI tract is also known as afferent innervation or gut-brain axis and arises from sensory ganglia residing in the nodose ganglia (NG) and dorsal root ganglia, specifically the thoracolumbar and lumbosacral DRG (Figure 5, p 40).

A main characteristic of dorsal root ganglia neurons is their enormous heterogeneity. In early studies, sensory neurons were classified according to their cell diameter/cell size and conduction velocity. Large

[7] S components have a slower migration in chromatography columns that F (fast) components

diameter neurons possess myelinated axons and high conduction velocities (A fibres). Depending on the degree of myelination, A fibres are further subdivided into Aα which have the highest conduction velocity and innervate muscles. Aβ fibres constitute the mechanosensitive afferent of the skin and Aγ fibres innervate muscle spindles. Aδ and C fibres are thinly myelinated and unmyelinated fibres with slow conduction velocities that have been shown to mediate pain sensation and innervate the viscera. Based on their differential binding of the isolectin B_4, DRG neurons were classified at IB_4^+ non-peptidergic and IB_4^- peptidergic neurons. In this classification, nociceptors belong to the population of IB_4^- neurons because they frequently express CGRP and SP. Later studies found that these groups are not distinct and there is considerable overlap between them. Most recently, single cell RNA sequencing has been used to investigate DRG neuron heterogeneity. Based on cluster analyses, sensory neurons were segregated into at least 10 subpopulations based on the expression of receptors and neuropeptides as well as functions (Chiu et al., 2014; Li et al., 2016; Usoskin et al., 2014).

2.1.1 Types of afferents

Retrograde tracing techniques have revealed that intestinal afferent neurons are located in the thoracolumbar and lumbosacral (splanchnic, pelvic afferents) region of the spinal cord as well as in the nodose ganglion (vagal afferents). The innervation pathways are shown in Figure 5. Neurons with intestinal projections are of small to medium size (< 30 µm) and their processes are largely unmyelinated or lightly myelinated C- and Aδ-fibres (Blackshaw et al., 2007; Furness et al., 2013).

Intestinal afferents display a large functional diversity which arguably is more important than their anatomical origin. Some functional classes are thought to be concentrated in distinct anatomical regions. For example, pelvic afferents comprise a higher proportion of low threshold fibres than splanchnic afferents (Brierley et al., 2004). As their somatosensory relatives, intestinal afferents can be activated by a wide range of chemical and mechanical stimuli. Most of our current knowledge has been gained using afferent recording techniques from either intact or flat-sheet preparations of the colon and small intestine with attached splanchnic, pelvic or mesenteric nerve bundles.

In intact preparations, different populations of mechanosensitive fibres can be distinguished based on their response to ramp distension. The firing of low threshold mechanosensitive fibres increases during intraluminal pressures up to 10 mmHg while high threshold fibres only become activated at higher pressures (Rong et al., 2004). Wide dynamic range fibres are active throughout the distension whereas

mechano-insensitve ("silent") fibres cannot be activated by mechanical stimulation but may be recruited under pathophysiological conditions (Hibberd et al., 2016; Prato et al., 2017).

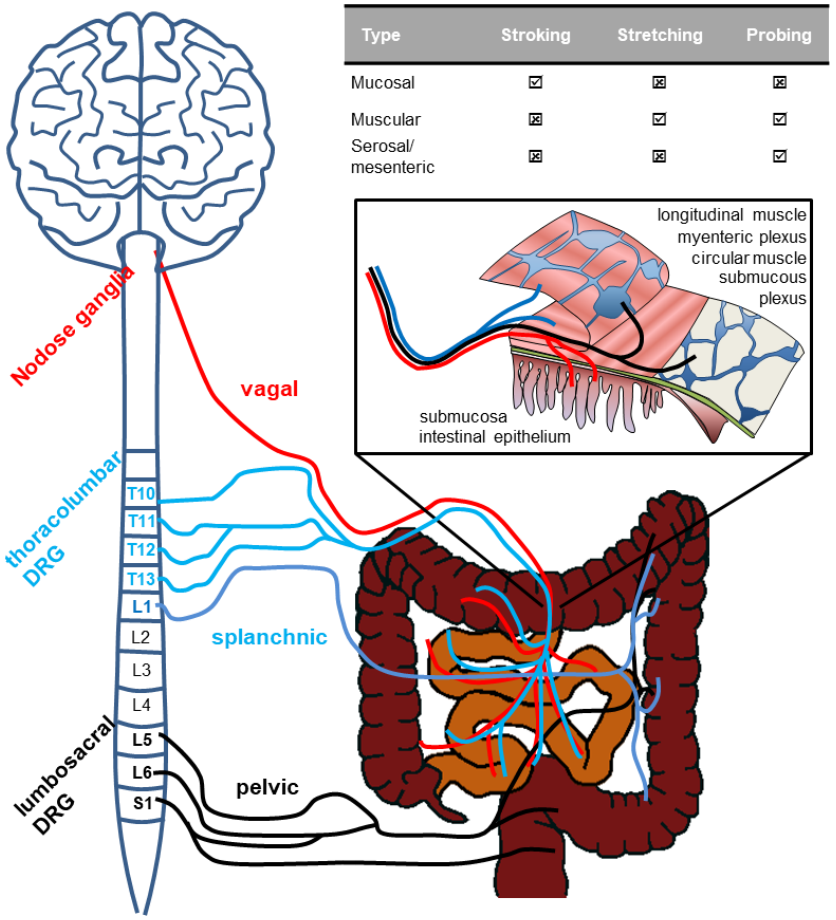


Figure 5: Extrinsic innervation of the GI tract. Neurons innervating the GI tract are located in the nodose ganglion and dorsal root ganglia. They dominate the innervation of the upper and lower gut respectively. Terminal endings in the different layers of the intestinal wall (insert) are activated by different stimuli (table insert). Figures combined and adapted from Furness et al., 20013, Hughes et al., 2009

Flat sheet preparations allow the application of three types of mechanical stimuli. The response pattern of isolated fibers to these stimuli can be used to distinguish different types of afferents (Brierley et al., 2005). Mucosal stroking (10-1000 mg), probing (70 mg–4 g) and circular stretch (1–5 g) activate mucosal, muscular/mucosal, mucosal, serosal and mesenteric fibres in the colon (Fig 4). A large proportion of fibers responding to stimuli with high intensity which are thought to be nociceptive, are present in splanchnic nerves (50 % mesenteric, 36 % serosal). In contrast, pelvic nerves contain significantly higher proportions of mucosal (23 %) and muscular (21 %) fibres. A similar approach has not been successful in the small intestine because of the limited tissue viability (Rong et al., 2004). In addition, a clear distinction between vagal, splanchnic and pelvic afferents is not possible in the upper

gut. All three pathways are present in the nerves of mesenteric arcades and contribute differently to the innervation of the proximal (vagal), mid (splanchnic) and distal (pelvic) intestine (Figure 5).

2.1.2 The terminal of afferent fibres

Sensory neurons express various membrane-bound proteins in their terminals that directly (ion channels) or indirectly (G protein-coupled receptors) affect membrane potential and nerve activity.

Mechanosensitivity

The precise mechanisms underlying the mechanosensitive response of sensory neurons are yet to be determined but it is believed that it is ion channels in the afferent terminal that can be directly opened as a result of membrane deformation. As putative candidates for mechanosensitive ion channels, transient receptor potential (TRP) A1 and TRPV4 have been suggested (Brierley, 2010). Genetic ablation of these genes reduces colonic responses to mechanical stimuli and visceral pain response to colorectal distension (Brierley et al., 2008, 2009; Mueller-Tribbensee et al., 2015). It has been shown that TRPV4 translates changes in the microenvironment (extracellular matrix) into an electrical signal suggesting that it is mechanosensitive itself and is not only involved in the transmission of the signal in the nerve terminal (Martinac and Poole, 2018).

Other channels that are thought to be inherently mechanosensitive include two members of the Piezo family, among which Piezo2 is specifically expressed in dorsal root ganglia neurons. Piezo2 is intracellularly connected to the cytoskeleton and therefore, changes of cell shape result in opening of the channels which allows the influx of cations. Others have also suggested that Piezo "senses" changes of the lipid bilayer that result from swelling or shrinking of the cell which may be induced by changes of osmolality (Cox et al., 2016; Wang et al., 2017).

Direct mechanosensitivity has also been described for ion channels that are involved in signal transduction regulating the cells excitability. These include voltage gated sodium channels (Drew, 2011; Morris and Juranka, 2007) and the two-pore-domain potassium channels TREK-1 and TRAAK[8] which are also activated by highly unsaturated fatty acids (Brohawn et al., 2014; Heurteaux et al., 2004).

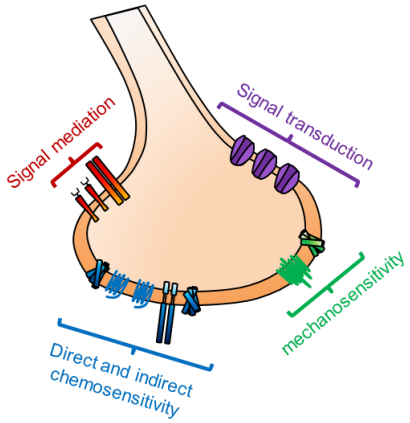
[8] Tandem of P domains in Weak Inward-rectifier K^+ channel (TWIK)-related K^+ channel, TREK; TWIK-Related Arachidonic Acid (AA)-stimulated K^+ channel, TRAAK (Lesage et al., 2000)

Chemosensitivity

Most small intestinal afferent nerves are also activated by chemical stimuli. In particular, mucosal afferents express receptors for a wide range of nutrient-related stimuli that induce afferent nerve firing either directly or indirectly. Sensory afferents can also be activated by a number of non-nutrient stimuli such as irritants and cell wall components of bacteria. A detailed overview of the receptors expressed on the afferent nerve terminal is shown in Figure 6.

Nutrient-sensing can be conferred via the expression of G protein-coupled receptors such as GPR41/43 (fatty acids), CaSR, potentially taste receptors, GPR119/120 (amino acids) and GPCR19 (bile acids) as well as nutrient transporters (sodium-glucose transporter, Sglt-1). It has remained challenging to determine whether visceral afferents are themselves sensitive to these stimuli. It is thought that indirect pathways also contribute to afferent nutrient-sensing (Husted et al., 2017; Williams et al., 2016).
Transient receptor potential (TRP) channels constitute a large family of multimodal receptors that can be activated by heat, irritants, cold etc. The TRPV1 and TRPA1 channels have a special role in visceral nociception (Brierley et al., 2010; Yu et al., 2015).
Tissue degradation and bacterial products can be sensed as described in section 1.1.5 via toll-like receptors and receptors for bacterial metabolites such as short chain fatty acids.

These receptors are also expressed in epithelial enteroendocrine cells (EEC) that release cholecystokinin (CCK), somatostatin, glucagon-like peptide (Glp-1), serotonin (5-HT) etc. upon stimulation (Cani et al., 2013; Ezcurra et al., 2013; Lund et al., 2018; Pais et al., 2016). These hormones can stimulate afferent fibers and thus, contribute indirectly to chemosensitivity. Bellono et al. (2017) convincingly demonstrated this concept in a recent study. The SCFA isovalerate was shown to induce 5-HT release from enterochromaffin cells (EC) and activated colonic afferents in a 5-HT receptor-dependent way when it was applied to the colonic mucosa. In line the concept that afferent nerve responses were the result of isovalerate-induced 5-HT release, colonic DRG neurons which are used as a surrogate for colonic afferents in-vitro, did not respond themselves to isovalerate (Bellono et al., 2017). Similar pathways have been suggested for CCK, peptide YY and Glp-1 which are released by different populations of endocrine cells (Gaisano et al., 2010; Hicks et al., 2002; Kreis et al., 1997).



receptor	ligand	function
Nutrient receptors		
TRP Channels - TRPV1 - TRPA1 - *TRPM8*	Detection of capsaicin, heat, low pH, 12-HpETE, 20-HETE, anandamide, LPA AITC, cinnamonaldehyde, cold, acrolein, 4-HNE, itch-sensation Cold, menthol	
Bacterial products - TLR	cell wall products, DNA, etc from bacteria, viruses, fungi	
Hormone receptors	Glp-1, CCK, oxytoxin, serotonin (5-HT3 and G protein-coupled receptors)	
PAR	Short peptides generated by proteolytic cleavage	Cleavage can be induced by serine proteases, thrombin and trypsin
Piezo2	Deformation of membrane and cell	mechanosensitivity
TRPV4	Extracellular matrix	mechanosensitivity
Cytokine receptors	Interleukins TNF-α histamine, prostaglandins	Induce direct activation of colonic afferents but also alter properties in inflammation
Neurotransmitter receptors		
- RAMP-1 - NK - *$GABA_A$,$GABA_B$* - P2X and P2Y - *opiod receptors* - *CB*	CGRP Neuokinins (Substance P) γ-amino butyric acid ATP dynorphins, enkephalins, endorphins, endomorphins and nociceptin cannabinoid receptors: anandamide, 2-arachidonoylglycerol	

Figure 6: Receptors and ion channels that are expressed in the afferent nerve terminal regulate excitability Adapted and expanded from Brierley et al., 2010, Chen et al., 2018. For references refer to text. Receptors in italic writing are inhibitory. HETE, hydroxyeicosatetraenoic acid; HpETE, hydroperoxy-ETE; HNE, hydroxynonenal; LTA, lysophosphatidic acid

Signal conduction

The diversity of sensory neurons is also represented in their expression of the ion channels that regulate the excitability of the cell. The initiation of action potentials depends on voltage gated sodium channels (VGSC, Na_V) whereas voltage-gated potassium channels contribute to the repolarisation phase. Here, main characteristics of these channels will be briefly introduced and we refer to previously published reviews for further details (Beyak and Vanner, 2005; Brierley et al., 2010; Erickson et al., 2018).

Na_V channels

VGSC are essential components present in the nerve terminal. They initiate action potential firing and are composed of a pore-forming α-subunit as well as auxiliary β-subunits that regulate the gating properties. To date, nine different α-subunits have been described and are traditionally divided into tetrodotoxin-sensitive ($Na_V1.1$, $Na_V1.2$, $Na_V1.3$, $Na_V1.4$, $Na_V1.6$, $Na_V1.7$) and TTX-resistant ($Na_V1.5$, $Na_V1.8$, $Na_V1.9$). TTX-resistant channels are more abundant in small diameter neurons that do not bind the isolectin B_4 (IB_4-positive) which is used to identify different classes of nociceptors. IB_4-negative neurons are thought to constitute peptidergic nociceptors that express various TTX-sensitive and TTX-resistant Na_V channels. Recent studies have shown that the majority of colon-innervating neurons expresses CGRP, i.e. are peptidergic (Hibberd et al., 2016), and they have also been found to express a number of TTX-resistant and TTX-sensitive channels (Hockley et al., 2017). Among them, $Na_V1.8$ and

$Na_V1.9$ were both present in about 90 % of the neurons and $Na_V1.7$ was equally highly present. In contrast, only 50-70 % of the neurons expressed $Na_V1.1$-1.3 and $Na_V1.4$ and $Na_V1.5$ were rarely detected. In a subsequent study, the link between the expression of neuropeptides and sodium channels was largely confirmed in colonic sensory neurons but non-peptidergic neurons also expressed TTX-sensitive $Na_V1.7$, $Na_V1.4$ and $Na_V1.2$ which indicates that the traditional classification used for somatosensory neurons may not apply to visceral neurons. Indeed, it is yet to be determined what constitutes a visceral nociceptor (Erickson et al., 2018).

Modulation of VGSC expression and activity has been implicated in somatic and visceral pain. For example, Osteen et al. (2016) describe that an agonist of the $Na_V1.1$ channel increased the sensitivity of high threshold colonic afferents and the excitability of colonic neurons (Osteen et al., 2016). Similarly, modulation of $Na_V1.6$-$Na_V1.8$ channel activation and inactivation properties by the seafood toxin ciguatoxin induces visceral pain (Inserra et al., 2017). On the contrary, the hyperexcitability of DRG neurons induced by infection with *Nippostrongylus brasiliensis* is significantly reduced in animals $Na_V1.8$ but not $Na_V1.9$ knock-out mice (Hillsley et al., 2006). These studies suggest that several isoforms contribute to visceral pain.

K_V channels

Voltage-gated potassium channels regulate inactivation patterns of sensory neurons and thus, blockage of these channels increases excitability. There are 40 pore-forming α-subunits in the human genome and these combine to functional heterotetramers. Electrophysiologically, K_V currents are classified into a 'transient' (A-type, I_A) and 'a sustained delayed rectifier' (I_K) type current based on their inactivation and pharmacological properties (Akins and McCleskey, 1993).

Sensory neurons express multiple K_V channels (Hockley et al., 2018; Jami et al., 2018) and a role of K_V channels in visceral sensitivity has been reported. Stewart et al. (2003) found that K_V currents were significantly reduced in guinea pigs after TNBS-ileitis and Qian et al. (2009) have shown that acetic acid-induced visceral hypersensitivity is linked to a decrease of $K_V4.3$ channel expression. In addition, the potassium channel opener retigabine reduces capsaicin-induced visceral pain (Hirano et al., 2007; Qian et al., 2009; Stewart et al., 2003).

Ca_V channels

Voltage-gated calcium channels play an important role in regulating neuronal excitability and neurotransmission. Ca_V channels are composed of a pore-forming α1-subunit and other subunits that are important for the regulation of the pore. There are 10 different α1 subunits that are sub-classified

into L-type (long lasting, $Ca_V1.x$), N-type (neuronal, $Ca_V2.x$) and T-type (transient, $Ca_V3.x$). Activation of Ca_V channels allows the entry of calcium which depolarises the membrane and also contributes to neurotransmitter release.

To date, only a limited number studies investigated the role of Ca_V channels in visceral (hyper-) sensitivity. It has been shown that $Ca_V3.2$ is upregulated in patients with Irritable bowel syndrome and that in mice, knock-out prevented the development of visceral hypersensitivity (Scanzi et al., 2016).

Sensitisation and desensitisation

The local environment affects neuronal excitability and this plasticity is also a hallmark feature of visceral neurons (Brierley and Linden, 2014). It is the result of changes of the membrane potential that are induced by modulation of the receptors and ion channels described above. A number of inflammation-associated stimuli such as TNF-α, prostaglandin E2 (PGE), 5-HT, bradykinin, ATP and NGF sensitise (depolarise) the nerve terminal to subsequent stimuli (Hockley et al., 2016; Maingret et al., 2008; Östman et al., 2008) whereas γ-amino butyric acid (GABA), nitric oxide (NO), opioids, (endo-) cannabinoids and related substances have a desensitising (hyperpolarising) effect (Hong et al., 2009; Petho and Reeh, 2012).

Receptors for these mediators are also expressed in visceral afferents and this has been implicated in visceral pain (Brusberg et al., 2009; Castro et al., 2017; Hughes et al., 2014). In nerve recording experiments it has been shown that incubation of colonic segments with these modulators alters the mechanosensitive response. These mediators can also induce spontaneous firing in mechanically sensitive but also in-sensitive colonic afferents (Brierley et al., 2005; Brunsden and Grundy, 1999; Campaniello et al., 2016).

These findings exemplify the complex interaction between signalling pathways at the afferent nerve terminal and that they are highly sensitive a variety of stimuli released within the intestinal wall.

2.1.3 Functions of sensory neurons

Depolarisation at sensory nerve terminal can directly induce neuropeptide release from vesicles close to the depolarisation site. Simultaneously, signal propagation initiates axon reflexes which induces transmitter release at the same or a collateral axon, and neurotransmission to secondary (CNS or inter-) neurons. By activating these pathways, peripheral stimuli can affect central centres involved in maintaining homeostasis, emotions and cognition.

Peripheral responses

Neurotransmitters released at the peripheral terminal of sensory nerves as a result of antidromic axon reflexes comprise the neuropeptides CGRP and SP as well as several other factors such as ATP, glutamate and BDNF. CGRP and SP are particularly well studied for their effects on peripheral organs. Here, we will focus on their modulatory function on the immune system.

It is becoming increasingly clear that neurotransmitters have remarkable effects on a number of immune cells. Immune cells express receptors for neuropeptides and *in-vitro* and *in-vivo* studies have demonstrated that incubation with CGRP and SP affects their phagocytic activity, production of antimicrobial substances, chemotaxis and differentiation. Furthermore, immune function is altered in animals where sensory signalling or neuropeptide signalling is disrupted (Mcmahon et al., 2015). For example, the CGRP receptor Ramp1 is expressed on macrophages, dendritic cells and T cells (Mikami et al., 2011, 2014). Binding of CGRP reduces TNF-α release which reduces T_H1 and increases T_H2 differentiation and this has been found to decrease inflammatory bowel conditions (Lai et al., 2017; Pinho-Ribeiro et al., 2017). On the contrary, activation of the Substance P receptor VPAC increases the release of chemokines and interleukins from neutrophils and other leukocytes which has an overall pro-inflammatory effect (Delgado et al., 2003; Sun et al., 2007). In addition to their direct effects on immune cells, both, CGRP and SP increase the permeability of blood vessel via their respective receptors on endothelial cells and thereby contribute to the recruitment of immune cells.

CNS responses

Activation of sensory neurons also induces synaptic transmission at central terminals. A detailed discussion about central connections of intestinal sensory neurons with deeper brain structures is beyond the scope of this thesis but because the functional outcomes such as behaviour, food intake and immune modulation are important and can be used as an indirect measure for gut-brain communication, they are described below.

Sensory neurons that innervate the intestine are located in DRG and NG which are particularly well connected to CNS centres that regulate basic body functions as well as pain, food intake and mood (Mayer, 2011). The projection pathways are shown in Fig 6 but in contrast to sensations from other parts of the body, visceral sensations are generally not consciously perceived. Extrinsic and intrinsic factors impact on these connections which can increase the awareness for visceral sensations. In

addition, these unconscious inputs play a major role in regulating behaviour and emotions and therefore, contribute to sickness behaviours during infection and disease (Dantzer, 2009). The involvement of the sensory neuronal inputs, in addition to inflammatory signalling, is supported by the close connection between infection or dysbiosis and CNS-associated diseases as well as studies showing that microbiota-induced changes of behaviour depend on intact sensory signalling (Bravo et al., 2011; Cawthon and de La Serre, 2018a; Clarke et al., 2013; O'Leary et al., 2018).

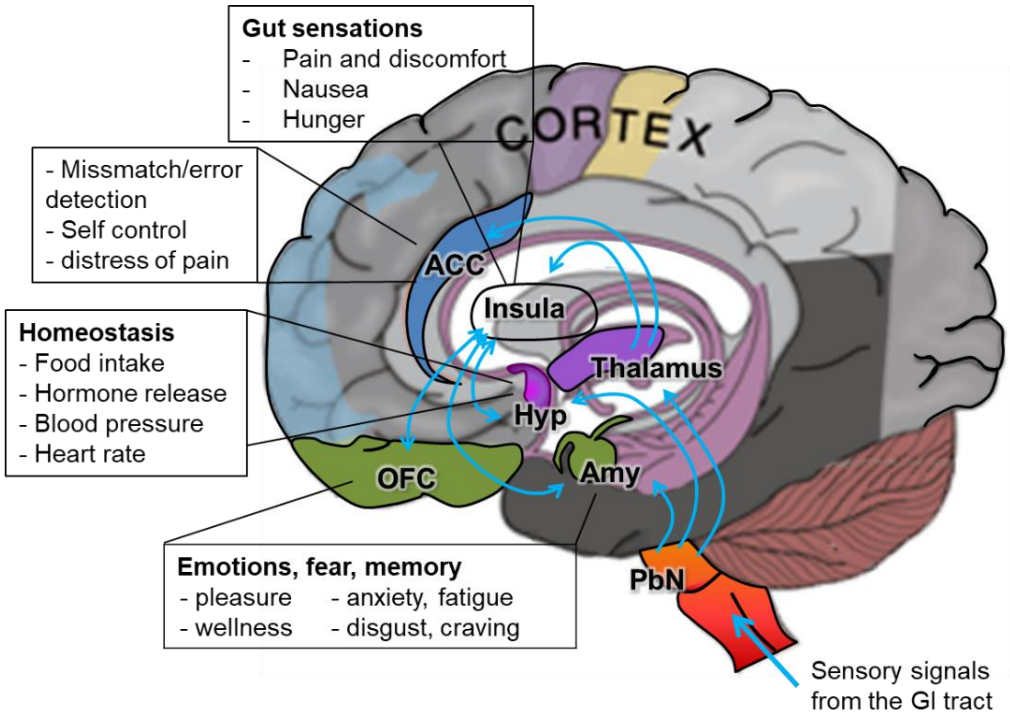


Figure 7: Sensory neurons modulate the activity of CNS structures. Vagal and spinal pathways converge in the parabrachial nucleus (PnN) which has projections to the thalamus, hypothalamus (Hyp) and the amygdala (Amy) which are implicated in gut sensations (through cortical projections to the insula), regulation of homeostatic body functions and emotions, respectively. ACC, anterior cingulate cortex; OFC, orbitofrontal cortex Figure conceptualised from Mayer et al., 2011.

2.2 Intrinsic Innervation

Sensory nerves of the PNS mediate gut-brain communication but it is the intrinsic nervous system that regulates enteric reflexes which are important for gastrointestinal motility, secretion, absorption and blood flow (Furness, 2012). One of the most striking findings that demonstrated the existence of a second brain in the gut is that it continues to generate coordinated activity when the connection to the brain is cut. This is indicative of non-centrally regulated contractile activity in the gut.

2.2.1 Components of the enteric nervous system

The general structure of the enteric nervous system shares many aspects of the brain. It is organised in two plexi of ganglia that are composed of neurons and glial cells. The submucosal plexus (Meissner's Plexus) is located between the mucosa and the muscle layer while the myenteric plexus (Auerbach's Plexus) is embedded in between the circular and longitudinal muscle layer (Figure 2, p 22). Enteric neurons are smaller than dorsal root ganglia neurons (Gabella, 1987; Santer and Baker, 1988) and subtypes of enteric neurons have traditionally been classified based on their morphology. Dogiel type I cells are characterised by one large (axon) and several short (dendrites) processes which usually do not branch to neighbouring ganglia. In contrast, Dogiel type II cells are oval-shaped and have long (axonal) processes that can project circumferentially. Type II cells are also generally larger than other cell types (Brehmer et al., 1999; Costa et al., 2000). However, this separation is not distinctively related to the function, electrophysiological properties and immunohistochemistry of the neurons.

Sensory neurons of the enteric nervous system (Intrinsic primary afferent neurons, IPAN) are thought to constitute the large cells with Dogiel type II morphology. In mice, these cells are immunopositive for choline acetyltransferase (ChAT), CGRP and neurofilament 145 kDa and constitute around 25 % of the neurons in the mouse myenteric plexus (Qu et al., 2008). No such cells were found in the submucous plexus of this species although IPANs have been described in the submucous plexus of larger animals (Mongardi Fantaguzzi et al., 2009). Electrophysiological recordings revealed that IPAN have a unique physiology which is characterised by Ca^{2+}- rather than Na^{+}-driven action potential and a long after-hyperpolarisation (Furness, 2000). IPAN respond to mechanical and chemical stimulation and release CGRP and ACh in mice as well as tachykinin in other species (Brookes, 2001; Furness, 2012; Kugler et al., 2018). In agreement with the immune-histological data, no neurons with AH physiology were found in the submucous plexus of mice (Wong et al., 2008).

Efferent neurons of the ENS are present in the myenteric and submucosal plexus and are mostly small uniaxonal neurons (Dogiel type I) that do not display the prolonged after-hyperpolaristion in patch clamping experiments (S-neurons). Stimulation by interneurons or IPANs induces the release of neurotransmitters such as nitric oxide (NO), ACh, vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), serotonin (5-HT), somatostatin or combinations of those. In mice, NO and VIP are produced by inhibitory muscle motor neurons in the myenteric plexus as well as vaso- and secretomotor neurons in the submucous plexus. In contrast, ACh is the main excitatory transmitter that induces contraction of smooth muscle and secretion (Furness, 2012; Mongardi Fantaguzzi et al., 2009; Qu et al., 2008).

Interneurons are a relatively small proportion of enteric neurons that project either orally (ascending interneurons) or anally (descending interneurons). They have been found in the myenteric plexus and are important for the generation and control of motor and secretomotor reflexes. Ascending interneurons are cholinergic while descending neurons express a variety of neurotransmitters that can be both excitatory and inhibitory (Brookes, 2001; Furness, 2000).

The ENS also constitutes a large number of enteric glia cells that do in fact outnumber enteric neurons. They comprise cells with divers morphology and marker protein (S100, GFAP, NTPdase, proteolipid protein) expression (Gulbransen and Sharkey, 2012). The particular functions of the different types of glia is still not entirely clear but there have been a number of recent studies that demonstrated their importance for the regulation of motility, secretion and neuronal survival. For example, deletion of PLP-1-positive glia has been shown to reduce gastrointestinal transit time in female mice but did not affect epithelial integrity. In contrast, virus-mediated elimination of GFAP-expressing enteric glia, increases epithelial permeability (Rao et al., 2017a) which could however also be a consequence of the systemic inflammation caused by the virus. Glial cells have also been shown to contribute to enteric neurogenesis after inflammation. Experimental colitis induces the differentiation of Sox2- and PLP-1-expressing glial into neurons and the proportion of Sox2-positive neurons is higher in patients with Crohn's disease compared to controls suggesting a glial origin (Belkind-Gerson et al., 2017).

2.2.2 Regulation of Motility

The enteric nervous system is the major regulator of gastrointestinal motility. Although the CNS is able to adjust motility to the overall state of the body via acetylcholine and noradrenaline, it is the ENS that coordinates and controls the contraction patterns of the GI tract. Therefore, modulation of ENS activity or function as seen in diseases such as Hirschsprung's is associated with severe motor dysfunction and constipation or diarrhoea (Furness, 2012).

The complex motility patterns generated by the activity of the ENS requires the coordinated contraction and relaxation of circular and longitudinal and circular muscle (Chen et al., 2013a; Smith et al., 2014). Muscle contraction is initiated by an increase of intracellular Ca^{2+} (Kuo and Ehrlich, 2015). This activates the Ca^{2+}-dependent myosin light-chain kinase (MLCK) which slides actin and myosin under ATP hydrolysis. In the circular muscle, Ca^{2+} is released from the sarcoplasmic reticulum whereas

contraction of longitudinal muscles requires Ca^{2+} entry from the interstitium (Murthy, 2006). Relaxation can be initiated by the neurotransmitter NO which is either released from enteric neurons or generated through VIP-induced eNOS activation, and GPCR-mediated signalling. They activate the protein kinases PKA and PKG that increase the activity of calcium pumps and MLC phosphatases which reduces intracellular Ca^{2+} MLCK activity (Kuo and Ehrlich, 2015).

The GI tract displays contractile activity even in the absence of ENS activity. Myogenic slow waves or "ripples" consist of rhythmic contractions that propagate orally and anally (Chen et al., 2013a; Costa et al., 2013) and are the result of small fluctuations of the muscle cell's membrane potential. Those are initiated and coordinated by Interstitial Cells of Cajal (ICC) located on the border of the myenteric plexus as well as the muscle layers (Hennig et al., 2010). The pacemaker potential is initiated by the release of Ca^{2+} from the smooth endoplasmatic reticulum through IP_3 receptors. This opens a Ca^{2+}-dependent Ca^{2+}-transporter in closely associated mitochondria and allows the influx of Ca^{2+}. Local Ca^{2+}-concentration decreases below baseline and releases the Ca^{2+}-block from a non-selective cation channel at the plasma membrane. Channel opening allows ion influx which drives the depolarisation of ICC and propagates to other ICC and muscle cells via gap junctions (Sanders et al., 2006).

In contrast to these local contractions, neuronal activity is required for complex motility patterns such as migrating motor complexes (MMC). Indeed, blocking neuronal transmission using TTX or lidocaine completely abolishes MMC and induction of muscle contraction with carbachol is not sufficient to recover MMC (Costa et al., 2013). Although the precise neuronal events that contribute to MMC are still being investigated (Spencer et al., 2005), it is thought that activation of sensory neurons (IPAN) is the first event leading to MMC (Costa et al., 2000). IPANs are particularly sensitive to deformation induced by pressure application or substrate stretch i.e. stress that induces a change of the length of the membrane and volume of the cell. Shear stress (parallel to the neuron) is not a potent stimulus (Kugler et al., 2018). They are also activated by serotonin which is released by enteroendocrine cells (EC) in response to chemical and mechanical stimulation. However, the involvement of epithelial serotonin in MMC initiation remains unclear because mice lacking the serotonin-producing enzyme TPH1 in the mucosa or removal or the mucosa does not prevent MMC (Li et al., 2011). It is therefore suggested that neurons might be the primary source of 5-HT and that 5-HT is involved in the propagation rather than the initiation of MMC (Heredia et al., 2013; Smith et al., 2014). Upon IPAN activation, the enteric reflex induces coordinated motility patterns through interneuron-mediated activation of both excitatory and inhibitory motoneurons. This results in muscle contraction at the oral

and relaxation in the anal direction respectively (Costa et al., 2000). Prior to contraction, a short depolarisation/relaxation period can be observed in electrical/tension recordings from muscle which is interpreted as the relief of the tonic NO-mediated inhibition of contractile activity but studies using nNOS-KO or inhibitors of nNOS remain inconclusive with regard to the requirement of disinhibition for the initiation or propagation of MMC (Brierley et al., 2001; Dickson et al., 2010; Smith et al., 2014).

2.2.3 Regulation of Secretion

The ENS also contributes to the regulation of secretion from epithelial cells. Constitutive secretion of ions and water from enterocytes compensates the nutrient-induced water and sodium transport across the epithelium. During the absorption process, monosaccharides, amino acids and fatty acids are taken up using passive or active (Na^+-coupled) transporters on the apical membrane which increases intracellular Na^+ and H_2O follows passively (Thiagarajah et al., 2015). On the basolateral membrane, 3 Na^+ are exchanged for 2 K^+ (Na^+/K^+-ATPase) to reduce Na^+ concentrations and this drives the uptake of Cl^-. Chloride and bicarbonate are secreted on the apical surface via CaCC and CFTR (Ca^{2+}-activated Cl^- channel; cystic fibrosis transmembrane regulator) and are followed by H_2O (Schulzke et al., 2014)

Secretomotoneurons of the enteric nervous system are located in the myenteric as well as the submucous plexus. They secrete either ACh, ATP and 5-HT or VIP and Pituitary adenylate cyclase-activating polypeptide (PACAP) which regulate secretion via receptor-mediated changes of Ca^{2+} and cAMP concentrations (Furness, 2012). For example, ACh via mACh3 receptors activates phospholipase C (PLC) via $G_{\alpha q}$. The subsequent increase of $[Ca^{2+}]_i$ activates CaCC-induced secretion. In contrast, other secretagogues such as VIP increase $[cAMP]_i$ via G_{α_s} and this directly regulated the activity of CFTR and Na^+/K^+-ATPase (Hirota and McKay, 2006).

The specialised epithelial cells involved in the secretion of gut hormones (EECs), antimicrobial peptides (Paneth cells) and mucus (Goblet cells) are also responsive to enteric neurotransmitters. This is particularly important during inflammation where acetylcholine can be released from enteric but also parasympathetic extrinsic neurons upon recognition of epithelial damage or bacterial invasion (Yoo and Mazmanian, 2017).

3 Do bacteria interact with neurons?

In the previous sections, I have described that intestinal bacteria have important functions for physiology and that host cells can detect a range of bacteria-related produces (section 1). Additionally, it was described that sensory neurons of the PNS and ENS respond to environmental stimuli and their activity is important for sensation, behaviour, mood and enteric reflexes (section 2). Considering the close association between bacteria and neurons in the GI tract, surprisingly the possibility of a direct interaction as only recently been considered. In the following, we will present evidence from the literature supporting this idea.

3.1 Bacterial products and intestinal neurons

Most studies investigating host-bacteria interactions have focussed on the long term effects of probiotic bacteria on neuronal excitability. While changes of neuronal activity in these studies could be indicative of a direct interaction, they may also be a consequence of a modulation of the inflammatory or metabolic status. In contrast, relatively few studies investigated the question whether bacteria and intestinal neurons interact directly and those have primarily tested mediators from commensal or probiotic bacteria.

Our group previously reported that lipopolysaccharide (LPS) from *Salmonella typhimurium* but not *Escherichia coli* (026:B6) increases spontaneous afferent nerve activity when it was perfused through the small intestinal lumen of pentobarbitone-anaesthetized rats (Liu et al., 2009). Others confirmed an excitatory effect of LPS (*E. coli* NLM28) on sensory neurons in patch-clamp recordings from colon-innervating dorsal root ganglia neurons (Ochoa-Cortes et al., 2010). With regard to enteric neurons, Mao et al. (2013) found that application of *Lactobacillus rhamnosus*, *Bacteroides fragilis* as well as the *B. fragilis* polysaccharide A spontaneously increases excitability of myenteric neurons (Mao et al., 2013). The same group also reported a decrease of jejunal motility by live *L. rhamnosus* but not heat-killed bacteria or *L. salivarius* (Wang et al., 2010; Wu et al., 2013).

Other studies have investigated whether mediators present in supernatants from patients with diseases that are linked to infection and dysbiosis affected neuronal function. Indeed, faecal supernatants from IBS-D patients activate DRG, sensory nerves and ENS neurons. This excitatory effect of supernatants depended on the expression of host protease-activated receptors (Buhner et al., 2009, 2018; Valdez-Morales et al., 2013b). In addition, others found that it is cytokines (TNF-α and IL-6) that are involved

in disease-induced hypersensitivity (Hughes et al., 2014; Ibeakanma and Vanner, 2010). Thus, although these supernatants may have also contained bacterial substances, in these studies it was host-derived substances that are responsible for the higher degree of neuronal activity in patients.

In contrast, two recent studies have used supernatants from bacterial cultures. The first study incubated colon-innervating dorsal root ganglia neurons with supernatants from a mixture of 33 commensal bacteria isolated from a healthy donor faecal sample and this decreased their excitability (rheobase and action potential firing). Heat-treatment and protease inhibition counteracted the supernatant's activity but the researchers did not further investigate whether it was indeed bacterial proteases that reduced neuronal excitability through PAR-4 (Sessenwein et al., 2017). The second study found that supernatants from *Escherichia coli* strain Nissle 1917 contain a GABA-related lipopeptide that decreassd Capsaicin- and GPRC-induced calcium influx in dorsal root ganglia neurons as well as visceromotor responses to colorectal distension dependent on the $GABA_B$ receptor. In this study however, the isolated substances but not the *E. coli* supernatants were used which provides a more mechanistic approach but ignores the presence of other potential neuromodulators in the supernatants (Pérez-Berezo et al., 2017). These studies suggest that bacterial substances present in supernatants from bacteria have neuromodulatory activities.

Studies using sensory nerve ablation or inhibition of neuropeptide signalling provide further indications for a bacteria-neuron interaction. In these studies, gastroenteritis symptoms (loss of appetite, inflammation-associated pain, social isolation) were reduced in animals with abrogated sensory innervation, interventions with antagonists for neuropeptides released from sensory nerves or knock-out mice. For example, vagotomy has been found to prevent neuronal activation in the brain (as measure with cFos) during *Campylobacter jejuni* infection (Goehler et al., 2005, 2008) and Substance P antagonists or knock-out improves disease severity in colitis models. These results support that the gut-brain axis contributes to symptom generation under these conditions but it is a direct interaction of the bacteria and sensory nerves that is reduced by these interventions (Lai et al., 2017).

Overall, the current literature suggests that neurons can be activated by bacterial substances and also that this interaction may contribute to the symptoms and long-term effects of neuronal activity after infection with pathogenic bacteria. This is in contrast to the paradigm that infection-associated release of inflammatory cytokines is solely responsible.

3.2 Pathogenic bacteria and neurons

The concept of inflammation-independent interaction of bacteria and neurons has also emerged from studies of extra-intestinal organs. In 2013, Chiu et al. (2013) showed that virulence factors produced by pathogenic *S. aureus* mediated spontaneous nocifensive behaviour in wild-type and immune-deficient mice. These virulence factor also increased DRG neuron excitability and induced calcium transients (Chiu et al., 2013). In line with a direct bacteria-neuron interaction, Meseguer et al. (2014) showed that LPS application had similar excitatory effects on NG and trigeminal neurons and intra-plantar injection of *E. coli* LPS induced pain and mechanical hypersensitivity (Meseguer et al., 2014).

Other bacteria that produce potent neurotoxins are *Clostridium botulinum* and *C. tetani*. In contrast to the excitatory effect of staphylococcal virulence factors and LPS, botulinum and tetanus toxins reduce hyperexcitability by interfering with neurotransmitter release (Chiu, 2018). Another bacterial compound, myolactone that is produced by *Mycobacterium ulcerans*, was also found to have analgesic rather than algesic properties which were mediated through hyperpolarisation of DRG neurons via angiotensin receptors (Song et al., 2017).

These findings support the idea that bacteria can directly modulate neuronal activity. They also suggest that, in the context of infection, neuroinflammation is not only, as traditionally thought, mediated by the cytokines that are released as a consequence of recognition of bacteria by the immune system. Rather, neuroinflammation could be induced by bacterial mediators themselves. The fact that some bacteria produce inhibitory mediators opens the question what the role of neuromodulation in the context of infection might be.

3.3 Changes of the microbiome are linked to chronic diseases with neuronal dysfunction

In this section, evidence for a direct interaction between bacteria and neurons was presented. In addition, several chronic diseases are associated with symptoms of neuronal dysfunction on the one hand and intestinal dysbiosis and/or infection on the other hand. This supports the idea that bacteria play a role in the pathogenesis of diseases and that bacterial products may be able to directly modulate neuronal activity. In the following, a few of these diseases will be exemplified.

3.3.1 Obesity

Obesity is defined as an abnormal accumulation of fat that presents a risk to health (www.who.int) which usually results from excessive energy intake combined with little expenditure. Afferent nerves contribute to the hypothalamic regulation of food intake and consistent with the idea that intestinal sensory signalling plays a role in obesity, vagotomy has been shown to reverse some metabolic alterations in obese rats (Balbo et al., 2016). A link between obesity and the microbiome has also been described: Microbiome composition is different between lean and obese twins (Turnbaugh et al., 2009), weight loss in obese humans is associated with a re-shaping of the microbiome (Ley et al., 2006), transfer of an "obese microbiome" into germfree mice increases weight and worsens glucose tolerance (Ridaura et al., 2013), genetically obese mice (ob/ob mice) display an altered microbiome (Turnbaugh et al., 2006), germ-free mice do not become obese (Bäckhed et al., 2004a).

With regard to distinct effects of obesity on the microbiome, Turnbaugh et al. (2009) first reported a lower abundance of Bacteroidetes and a higher abundance of Actinobacteria and Firmicutes in obese compared to non-obese twins (Turnbaugh et al., 2009). At the genus levels, this was associated with an enrichment of *Alistipes* and *Barnesiella* (Bacteroidetes) in controls and *Collinsella* (Actinobacteria) in obese twins (Duvallet et al., 2017). However, other studies report an increase of members of the Bacteroidetes phylum (*Prevotella*, *Porphyromonas*) in obese individuals (Zhang et al., 2009). Also with regard to Firmicutes, the abundance of different genera was altered into opposing directions which indicates that broad classification of bacteria at phylum level may not be sufficient to distinguish obese and non-obese individuals. These partially contradictory findings may be related to the multifactorial pathogenesis of obesity. Differently composed diets can lead to an increase of body weight and fat mass. The dietary intake and origin of macronutrients has a great impact on the microbiome and therefore, it is not surprising that cross-comparisons of studies have not identified a bacterial signature linked to obesity in humans (Sze and Schloss, 2016).

In animal models, diet can be better controlled but the findings with regard to the microbiome are also still inconclusive. In line with human studies, a decrease of Bacteroidetes has been observed in obese animals (Turnbaugh et al., 2006). A relative increase of Firmicutes has also been found some studies but similar to human studies, this was not consistent at genus level. For example, *Lactobacillaceae* (Firmicutes) were found to decrease with obesity induced by a high fat diet in two studies (Cani et al., 2008; Fleissner et al., 2010). Cani et al. also found that a decrease of *Bifidobacterium* (Actinobacteria) and *Enterobacteria* (Proteobacteria) was linked to obesity (Cani et al., 2007, 2008).

3.3.2 Functional gastrointestinal diseases – IBS

Functional gastrointestinal diseases are a pathology associated with a Western lifestyle and include functional dyspepsia, Irritable Bowel Syndrome (IBS). IBS affects approximately 10-20 % of the general population and is diagnosed based on Rome IV criteria[9]. Based on the predominant symptom, IBS patients are classified into diarrhoea- or constipation-predominant (IBS-D or IBS-C) or a mixed type IBS (Lacy and Patel, 2017). The pathogenesis is still incompletely understood but chronic GI pain is indicative of visceral hypersensitivity and indeed, interventions targeting gut sensory signalling are being developed as potential treatment options (Lacy et al., 2016). A possible link between IBS and the microbiome has also been suggested: Microbiomes of patients and healthy controls are different, the Bristol stool scale which is used for IBS diagnosis, is correlated with microbiome composition (Falony et al., 2016; Liu et al., 2017; Malinen et al., 2005; Tap et al., 2017). It has also been found that in a subgroup patients (post-infective IBS), symptoms develop after gastroenteritis (Spiller, 2003).

It is still a matter of debate whether IBS is associated with changes of the microbiome composition at a phylum level. For example, Tap et al. (2017) observed a reduced prevalence of the Prevotella enterotype in IBS patients. Their study also suggested that an increase of *Bacteroides* is linked to symptom severity which is in line with an overall increase of the Bacteroides in several IBS populations (Jalanka-Tuovinen et al., 2014; Parkes et al., 2012; Pozuelo et al., 2015). In contrast, others find that Firmicutes or taxa belonging to the Firmicutes phylum are more abundant in IBS compared to control (Jeffery et al., 2012; Rajilić-Stojanović et al., 2011). Members of the Lachnospiraceae family (Firmicutes) for example were associated with IBS-D (De Palma et al., 2017) or IBS-C (Zeber-Lubecka et al., 2016) and *Ruminococcus* (Firmicutes) with all IBS subgroups (Rajilić-Stojanović et al., 2011). There is particular dispute about the role of the probiotic bacteria *Lactobacillus* (Firmicutes) and *Bifidobacterium* (Actinobacteria) in IBS.

On the whole, it appears that the microbiome is affected differently in the IBS subtypes (Pozuelo et al., 2015; Tap et al., 2017) or is linked to other comorbidities associated with IBS including anxiety, depression, food allergies, bloating, flatulence, pain and chronic inflammation (Bennet et al., 2015; Liu et al., 2017). This suggests that subgroup analysis and deep phenotyping might provide new insights into the role of the microbiota and an instrument to develop targeted treatment strategies. Unspecific replacement (transplantation) of a "healthy" microbiota has also been suggested (Bennet et al., 2017).

[9] Rome IV defines IBS as recurrent abdominal pain, on average at least 1 day per week in the last 3 months, that is related to defecation and/or associated with a change in frequency of stool and/or associated with a change in form (appearance) of stool (2 or these three for the last three month). (Lacy et al., 2016)

3.3.3 Diseases of the Central Nervous System

The composition of the microbiota undergoes critical changes at times where neuronal function is particularly sensitive (Borre et al., 2014). This and the intricate interaction between bacteria and their metabolites with the host neuroendocrine system indicate that neuronal dysfunction could be a consequence of intestinal dysbiosis. Brain functions (behaviour, memory, emotion) may be particularly sensitive to alterations of the microbiome because sensory signalling allows a direct communication between intestine and the CNS.

Autism and Schizophrenia

Autism and Schizophrenia are both neurodevelopmental disorders that are characterised by profound changes of behaviour. Autistic children display repetitive behaviours and a reduced social interaction/ communication compared to non-autistic children. Symptoms used for the diagnosis of schizophrenia include affected information processing (delusions, hallucinations, affective flattening), anxiety, social isolation and lack of motivation. These phenotypes resemble the behavioural changes that can be observed in germfree mice which suggested that the microbiota contributes to both phenotypes.

The microbiome of autistic children differs from non-autistic children with regard to *Prevotella* (Bacteroidetes) and *Clostridium* (Firmicutes) as well as *Lactobacillus* (Firmicutes). The former was found to be less abundant (Kang et al., 2013) and the latter enriched in autistic children (Song et al., 2004; Tomova et al., 2015; Williams et al., 2011). Because of the saccharolytic metabolism of these Firmicutes species, this is also in line with observations higher SFCA levels (Wang et al., 2012). However, decreases of other members of the *Firmicutes* phylum (*Coprococcus*, *Veillonellaceae*, *Eubacterium*) as well as an increased abundance of Proteobacteria have also been reported (De Angelis et al., 2013; Kang et al., 2013; Williams et al., 2011). In an animal model of autism, intraperitoneal injection of the TLR3 ligand poly(I:C) induces deficits in communicative, repetitive, anxiety-like, sensorimotor, sociability and social preference behaviours and was associated with changes of the microbiome. A higher abundance of the *Porphyromonadaceae*, *Prevotellaceae* and unclassified *Bacteriodales* (Bacteroidetes) as well as *Lachnospiraceae* (Firmicutes) was observed. Simultaneously, other members of the Firmicutes were reduced (Hsiao et al., 2013). Although these findings were mostly opposite to what has been described above for humans, this study also found that a bacterial metabolite (4-ethylphenylsulfate, 4EPS) which is related to p-cresol (4-methylphenol), a marker for autism in humans, was elevated in autistic mice. This indicates that it is the bacterial metabolism rather than individual bacteria that could be targeted in translational approaches (Kushak and Winter, 2018).

With regard to schizophrenia, it is known that gastrointestinal infection and low grade intestinal inflammation are risk factors for the development of this disease (Dickerson et al., 2017). In addition, antibodies against intestinal fungi or parasites such as *Saccharomyces*, *Candida* or *Toxoplasma* are frequently detected in the plasma of schizophrenia patients. Although this indicates a link between schizophrenia and microbiome, there have only been few studies that addressed this question (Kelly et al., 2017). Schwartz et al. (2018) recently published an observational study showing that *Lactobacillus* was strongly associated with schizophrenia symptoms and that *Bacteroides* and *Ruminococcus* were more prevalent in the healthy study population (Schwarz et al., 2016).

Alzheimer's and Parkinson's Disease

Alzheimer's disease (AD) and Parkinson's disease (PD) are diseases of the CNS that predominantly affect the older population. AD is characterised by the extracellular deposition of amyloid-β (Aβ) peptides and the intracellular accumulation of neurofibrillary tangles. Aβ peptides are produced constitutively by various cell types including intestinal epithelial cells and neurons (Puig et al., 2015). Intestinal Aβ can be transported to the brain but also accumulates and forms amyloid plaques in the intestine. Infection is known to potentiate AD pathology (Kountouras et al., 2006; Sy et al., 2011) and brains of AD patients display increased accumulation of LPS (Sheng et al., 2003; Zhao et al., 2017) which indicates that bacteria might be involved in AD pathogenesis. A particular link to gastrointestinal bacteria is suggested by the observation that AD-like symptoms are reduced in germfree mice (Harach et al., 2017) and an increased abundance of proinflammatory gut bacteria in AD (Cattaneo et al., 2017). Furthermore, Vogt et al. found additional differences between the AD and healthy microbiome including less Firmicutes (*Dialister*, *Turicibacter*, *Haemophilus*, *Christensenellaceae*, *Lactococcus*, *Oscillospira*, *Lachnospira*, *Clostridiales*, *Ruminococcus* and most *Lachnospiraceae*), more *Bacteroides* and *Akkermansia* (Vogt et al., 2017).

Neuronally included Lewy bodies, aggregates of phosphorylated α-synuclein, are the hallmark feature of PD, a neurodegenerative disease that is most frequently linked to motor-deficiencies. Prior to those motorsymptoms however, PD patients experience gastrointestinal symptoms (Nair et al., 2018). In PD patients, α-synuclein deposition in enteric neurons has been described as a consequence of oxidative stress caused by the translocation of bacterial products under "leaky gut" conditions (Forsyth et al., 2011). A reduction of the intestinal barrier could be linked to changes of the microbiome composition (section 1.1.2) and indeed a difference between PD patient's and healthy control's microbiome has been

described (Parashar and Udayabanu, 2017). Those studies report a decrease of *Prevotella* and butyrate-producing bacteria from the Firmicutes phylum. In contrast, genes for proteolytic activity, lipopolysaccharide biosynthesis and bacterial secretion were higher in PD patients which is in line with a higher pro-inflammatory capacity (Keshavarzian et al., 2015; Scheperjans et al., 2015; Unger et al., 2016).

In addition to the diseases discussed above, an altered microbiome composition has also been linked to anxiety and depression (Burokas et al., 2017b; Lach et al., 2018). The pathology of these disorders less clear and anxious or depressive behaviours are often used for the diagnosis of other CNS diseases. Therefore, the reader is referred to previously published reviews for a further discussion of the microbiota in these circumstances (Cryan and Dinan, 2012; Winter et al., 2018; Yarandi et al., 2016).

4 Aims of this thesis

In this thesis, the hypothesis that soluble mediators from bacteria activate intestinal sensory signalling was investigated. Because previous studies have shown that *Staphylococcus aureus* induces pain and neuronal activation, this opportunistic pathogen was chosen for a "proof-of-principle" study. The objectives were to

(1) Establish whether soluble mediators released by *S. aureus* affect afferent nerve activity in ex-vivo preparations of the intestine and identify which mediators contribute to potential effects

(2) Understand the mechanisms underlying neuromodulation by those mediators in cell-based assays

(3) Investigate a potential modulation of the enteric nervous system by *S. aureus* mediators through assessing their effect on intestinal function

(4) Perform afferent nerve recordings from small intestinal afferents in a mouse model of visceral hypersensitivity and test whether sensitivity to bacterial mediators changes as a consequence of the inflammatory insult.

CHAPTER II

Soluble mediators from *Staphylococcus aureus* modulate intestinal afferent activity and intestinal functions

***ABSTRACT*:**

Commensal bacteria have important functions for human health. On the contrary, pathogenic bacteria can induce severe acute illness and are associated with chronic disease. It is emerging that neuromodulation contributes to the beneficial or detrimental effects of these bacteria. We tested this hypothesis for the pathogenic bacterium *Staphylococcus aureus*.

Supernatants were prepared from cultures of the *S. aureus* strain JE2 (SSA) and applied for 90 min to an *ex-vivo* preparation of afferent nerves innervating the distal small intestine that was distended to 30 mmHg every 15 min. SSA were perfused either through the organ bath or the intestinal lumen. The effect of SSA on intestinal function was investigated in Ussing chambers and recordings of colonic migrating motor complexes (MMC).

Bath application of SSA induced profound changes of afferent activity. As a 1 in 10 or 1 in 5 dilution, SSA (10 and 20 % v/v) transiently increased spontaneous discharge before nerve activity was significantly inhibited. SSA also remarkably reduced mechanosensitivity. Undiluted SSA applied to the intestinal lumen had a smaller excitatory effect and inhibited nerve activity only during late application where preparations had been continuously distended for 90 min prior to SA application. In Ussing chambers, we observed a strong increase of short circuit current when SSA were applied to the basolateral chamber whereas apical SSA had no effect. The increase of secretion was not sensitive to inhibitors of neuronal activity (tetrodotoxin, ωconotoxin, high concentration of capsaicin) or antagonists of serotonin (cilansetron) and ATP receptors (PPADS). Furthermore, bath-applied SSA reduced the amplitude of MMC whilst the frequency was not affected.

These findings show that pathogenic bacteria produce compounds that modulate the activity of neurons which contribute to symptoms of bacterial infection such as pain, discomfort, nausea and vomiting; and also submucosal targets to modulate intestinal functions which may be involved in diarrhoea symptoms.

1 Introduction

The gastrointestinal tract is colonised by large numbers of commensal bacteria. The intestinal microbiota outnumbers host cells and is essential for human health. These bacteria are able to metabolise nutrients that cannot be degraded by human enzymes and produce vitamins, short chain fatty acids as well as amino acids (Huttenhower et al., 2012), constitute a natural barrier for pathogenic bacteria (Kamada et al., 2013) and contribute to the development of the immune system and enteric nervous system (Hemarajata and Versalovic, 2013). Germfree animals display a number of behavioural abnormalities and need to be maintained under sterile conditions because of their deficient immune system (Lyte and Cryan, 2014). Although similar studies cannot be performed in humans, it is emerging that a dysbiotic microbiota is associated with numerous diseases. It is not clear yet what constitutes a "diseased" or "healthy" microbiome but an increase of bacteria with pathogenic potential has been frequently reported (Duvallet et al., 2017).

Changes of metabolism and immune activation are classically considered to underlie the beneficial effects of a healthy the microbiota (Cani et al., 2013). In this concept, a diseased microbiome releases metabolites with negative effects on health and induces over-activation of the immune system. It is also being suggested however that the microbiota modulates neuronal activity which may explain the close association between dysbiosis and CNS diseases.

The intestine is highly innervated by neurons of the peripheral and enteric nervous system (PNS, ENS) which are involved in gut-brain communication and the regulation of intestinal function (Furness et al., 2013). Peripheral and enteric neurons have been shown to express receptors for bacterial products (pattern recognition receptors, PRR) and respond to substances released from commensal bacteria and isolated substances such as lipopolysaccharide (Ochoa-Cortes et al., 2010; Sessenwein et al., 2017). Activation of enteric neurons by commensal bacteria may underlie their contribution to normal bowel habits whereas peripheral nerves ("gut-brain-axis") are important for the regulation of food intake and modulation of behaviour. Sensory peripheral nerves however also contribute to conscious sensations and pain. Thus, it would be detrimental if commensal bacteria profoundly activated these pathways (Brierley and Linden, 2014). In addition, over-activation of the ENS may lead to alterations of intestinal motility and secretion and this can result in diarrhoea or constipation. It is thus of utmost importance to understand how to distinguish between beneficial and harmful bacteria.

The barrier function of the epithelium it thought to contribute to this distinction. When intact, it prevents the tissue damage and immune-activation by bacteria. In contrast, under conditions where the barrier is compromised, commensal or more importantly pathogenic bacteria can translocate across the epithelium, disrupt tissue integrity and induce cytokine secretion from resident immune cells. Intestinal neurons can sense these signals and this is thought to underlie symptoms during infection (Campaniello et al., 2017). This concept of a purely indirect effect of pathogenic bacteria has been challenged recently in studies investigating skin infections (Chiu et al., 2013; Meseguer et al., 2014). Here, it was the direct interaction of bacterial mediators that causes acute pain sensation and hyperalgesia. This raises the possibility that intestinal afferents may also directly respond to signals from pathogenic bacteria.

Staphylococcus aureus is an opportunistic pathogen that is part of the healthy human microbiota. It colonises the nares of 30 % of the healthy population, the skin and the gastrointestinal tract particularly of young children (Boucher and Corey, 2008). It also constitutes a major threat for human health because of the emergence of antibiotic-resistant strains. Infection with pathogenic *S. aureus* or other pathogens induces spontaneous pain, nausea, vomiting and altered stool habits which severely impair patient's quality of life (Tong et al., 2015). Therefore, *S. aureus* is a good candidate to study the interaction of the host with commensal and pathogenic bacteria. Despite great efforts, it is still not entirely clear what defines commensal versus pathogenic strains of *S. aureus*. Most pathogenic strains secrete large amounts of various virulence factor that kill immune cells and degrade host-defence proteins which enables invasion and persistence in host tissue (Otto, 2013). Two secreted factors, α-haemolysin and formyl peptides, have also been implicated in an acute inflammation-independent pain response during infection with *S. aureus* (Chiu et al., 2013). This occurred within hours after infection and was thus different from the inflammatory pain that arises at later time points during infection as a consequence of immune activation. It has also been shown that the pain pathways may be important to modulate the inflammatory response (Baral et al., 2018).

In the current study, we have examined the effect of supernatants from *S. aureus* on sensory signalling from the gastrointestinal tract and associated secretory and motor functions. We describe a biphasic intestinal afferent response to bath applied-supernatant which is absent when applied luminally implicating a protective role for the intestinal epithelium.

2 Methods

Animals

In this study, adult male C57Bl/6 mice were used for afferent nerve and motility recordings. They were kept under standard husbandry conditions and sacrificed according to UK regulations (Animal Scientific Procedures Act, 1986) via overdose of isoflurane and cervical dislocation. A subset of experiments was performed in Adelaide (Australia) according to local regulations (supplementary methods, p 193).

Ussing chamber experiments were performed using Dunkin Hartley guinea-pigs (300-550 g, Harlan-Winkelmann, Germany). Those were maintained and humanely killed (cervical dislocation and exsanguination) in accordance with German ethical guidelines for animal experiments (TU Munich).

Supernatant preparation

Supernatants from *S. aureus* JE2 (SSA) were prepared using standard microbiological techniques (supplementary methods, p 198). Briefly, bacteria from bead stocks (-80 °C) were plated onto tryptic soy broth (TSB) agar plates under sterile conditions. They were grown at 37 °C overnight and maintained for maximal 14-21 d after plating (4 °C). Then, 50 mL TSB were inoculated with a single colony from these plates and cultured overnight at 37 °C on a rotary shaker. Optical density (OD) of the suspension culture was determined and diluted into a 500 mL TSB (2 L flask) to an OD of 0.150. These were incubated for 24 hours (37 °C, shaker). Cultures were centrifuged (5000 g, 10 min, 4 °C) and supernatants frozen (-20 °C) for later use. For Ussing Chamber experiments, supernatants were passed through a sterile filter and added into the recording chamber (Ussing chamber experiments) after thawing. In electrophysiology experiments, defrosted supernatants were diluted by volume with Krebs (20 %). For lower concentrations, proportions of SSA were replaced with TSB to account for vehicle-induced excitation (e. g. 10 % SSA + 10 % growth medium + 80 % Krebs) and maintain 20 % bacterial growth media (vehicle).

Afferent nerve recordings

The activity of nerves innervating the small intestine was recorded as described in previous studies (Nullens et al., 2016; Rong et al., 2004). A segment from the distal small intestine with the attached mesenteric arcade was isolated from the mouse. It was flushed with Krebs (composition in mM: NaCl 120, KCl 5.9, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 15.4, glucose 11.5, and $CaCl_2$ 1.2) and placed into a custom-build organ bath chamber. Surgical suture was used to tie the segment on both ends to a luminal perfusion system while the organ bath chamber was continuously perfused with warm

(33-35 °C) carbogenated Krebs solution. A three-way tab that was connected to the anal end of the perfusion system allowed to distend the tissue and measure intraluminal pressure. The nerve was dissected and placed into a recording electrode. Nerve activity as well as pressure measurements were amplified, filtered and acquired using the NeuroLog system and the Micro 1401 MKII interface with Spike2 software.

Spontaneous firing and mechanosensitivity were analysed as described in the supplementary methods (p 195) using Spike2 offline analysis and MicrosoftExcel®. Spontaneous nerve activity (NA) is expressed relative to baseline and distension responses were calculated as a percentage of the maximal increase of nerve activity during the control distension at 30 mmHg (ΔNA, [% control distension]). Low and high threshold components (LT and HT) were determined by analysing distension-induced increase at 15 and 30 mmHg respectively. For SSA bath application, bacterial growth medium (vehicle) was applied prior to SSA to account for vehicle-induced excitation that may affect each nerve differently. Alternatively, SSA were perfused through the lumen either directly after the preparation had stabilised (early application) or subsequent to 10 control distension (late application). Undiluted SSA (100 %) were used because nerve activity was not affected at lower concentrations (20 %) in preliminary experiments (not shown).

Intestinal motility and nerve recording from the proximal colon

Motility experiments were performed in analogy with the small intestinal nerve recording experiments with a 3-4 cm long segment anally from the caecum. It was perfused at 300 µL/min for maximal 30 min during which the nerve innervating the proximal colon was dissected and placed into the recording electrode. Then, the velocity of the perfusion pump was decreased and the outflow tab closed to allow accumulation of fluid inside to colonic lumen. When the pressure reached 3-4 mmHg, the colon started to contract and the perfusion was stopped. SSA or vehicle were bath-applied at a concentration of 5 % v/v (in Krebs) directly after the initial contractions for one hour, followed by a 30 min wash period. At 20 % v/v, vehicle profoundly reduced contractile activity and although this inhibitory effect was reversed during wash-off, we did not further pursue these experiments. Bethanechol (100 µM in Krebs, agonist of mAChR) was applied at the end of the experiment.

Contraction amplitude (maximum) and frequency were analysed during 15 min time intervals at the end of SSA or vehicle application and the wash period. The amplitude of contractions was also quantified during Bethanechol application using Spike2 software.

Ussing Chambers

Ussing Chamber experiments were performed using EasyMount Chambers (Physiologic Instruments, San Diego, USA) at the TU Munich. Mucosa-submucosa preparations from the distal small intestine of guinea pigs were isolated by removing the longitudinal muscle, the myenteric plexus and the circular muscle layer. During the dissection, tissue was perfused with ice-cold Krebs solution (in mM: 117 NaCl, 4.7 KCl, 1.2 $MgCl_2x6H_2O$, 1.2 NaH_2PO_4, 20 $NaHCO_3$,2.5 $CaCl_2x2$ $H_2O$and 11 glucose). The system was calibrated before each experiment and tissue mounted into the inserts (0.3 mm^2) of the recording chambers. Both half chambers were filled with 5 mL Krebs solution, continuously carbogenated (95 % O_2/5 % CO_2) and maintained at 35 °C. Tissue was allowed to stabilise for 45-60 min and short circuit currents (SCC) were recorded in voltage clamp mode using Ag/AgCl electrodes in KCl (Krueger et al., 2010). At the beginning and end of the experiment, tissue resistance was determined by switching to the open recording mode. Additional platin electrodes which were connected to a voltage stimulator (Grass SD-9, Astro-Med Inc., WestWarwick, USA), were used to generate and deliver an electrical field stimulus (EFS, 10 s pulses of 0.5 ms length, 10 Hz, 6 V) every 15 min.

Data was analysed using LabChart software and expressed relative to the SCC at the end of the stabilisation period i.e. baseline (fold BL_0 min) either in non-stimulated conditions or in the presence of EFS. Sterile-filtered supernatants were applied to either side of the recording chamber and the change of volume compensated by pipetting the same amount of Krebs solution into the other chamber. A sustained response was calculated by addition of individual time points as area under the curve (Fig S4)

Isolation of endogenous Staphylococci

To test whether the response magnitude to SSA was affected by the presence of endogenous Staphylococci, bacterial load was determined using Baird Parker plates (Oxoid) which selectively allow the growth of telliurite-resistant bacteria (Staphylococci). The luminal content was collected when the intestines were flushed in preparation for electrophysiological recordings, vortexted and pelleted by centrifugation (5000 g, 10 min). The pellet was weighted and re-suspended in 2x volume sterile PBS. Dilutions (1:3, 1:30, 1:200) were prepared and 20 µL plated onto agar plates. Plates were incubated overnight, photographed and colony-forming units (CFU) were counted at the lowest dilution that displayed individual colonies.

Statistical analysis

Data was analysed offline, processed and interpreted using Spike2 or LabChart, MicrosoftExcel® and SPSS statistics (version 23, IBM) respectively. For statistical analysis and graphical presentation

(median± interquartile range), data was transferred into GraphPad Prism® (version 7). Non-parametric statistical tests (Kruskall-Wallis test with Dunn's posthoc analysis, Mann-Whitney test) were used.

3 Results

3.1 Modulation of intestinal afferent sensitivity by soluble mediators from *Staphylococcus aureus*

Biphasic effect of SSA on spontaneous discharge

Small intestinal afferent spontaneous firing was significantly affected by bath application of supernatants of cultures from *S. aureus* (SSA). In the example trace of a nerve recording experiment using 20 % v/v SSA (Figure 1A), it can be seen that there was a biphasic afferent response with an initial excitation followed by profound inhibition which completely silenced the nerve firing. The SSA-induced increase of nerve activity was maximal at about 30 min of SSA application where nerve activity reached 214.3 % (median) of the firing at the start of the experiment baseline. Bath application of 20 % vehicle also increased afferent discharge but the degree of excitation was significantly smaller (148.7 % of baseline, $p = 0.006$ compared to SSA). In Figure 1A, it can also be seen that prolonged application of SSA reduced this excitation back to vehicle levels by about 60 min and inhibited spontaneous firing significantly below baseline levels (35.04 % of baseline firing, median) at the end of the experiment (Fig 1B1, Fig 1B2). In contrast, vehicle-induced excitation was maintained. This observation is consistent with SSA containing a number of mediators that cause excitation and/or inhibition of afferent firing.

Next we determined the extent to which excitation and inhibition were dependent upon mediator concentration by examining different dilutions of the supernatant. With 10 % v/v SSA the response profile was similar to that seen at 20% (Fig 1B1). Both, excitation and inhibition, were not significantly different to 20 % SSA. Bath-application of 5 % SSA induced a level of excitation that was comparable to 10 % and 20 % SSA but the inhibitory effect was significantly reduced (Fig 1B1 and 1B2). This indicates that the degree of excitation is not linked to the inhibitory effect of 20 % SSA and suggests that it is different components that mediate excitation and inhibition of afferent nerve discharge because the excitatory but not inhibitory mediators are already supramaximal at 1 in 20 dilution.

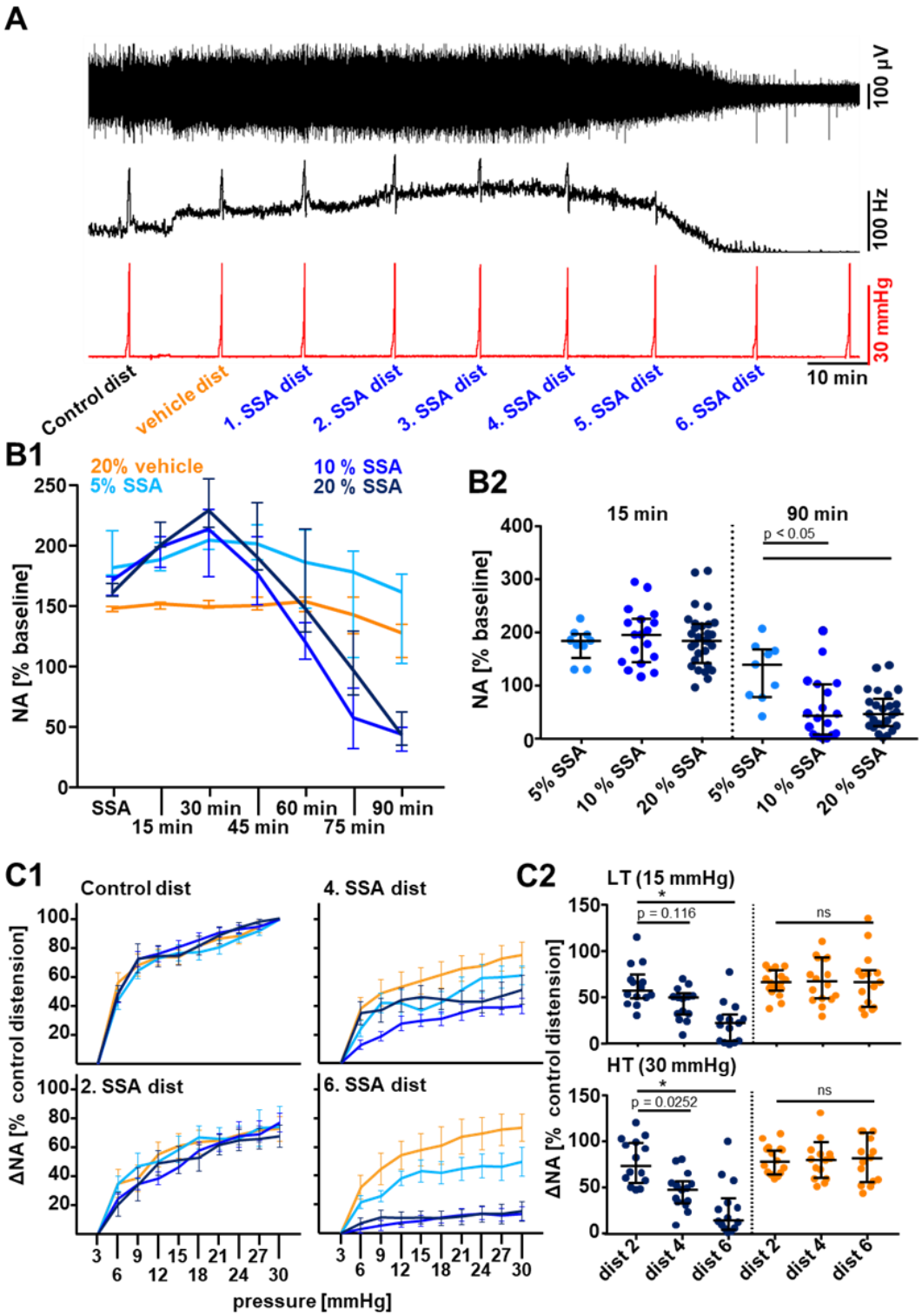


Figure 1: Bath application of supernatants from *S. aureus* (SSA) modulates intestinal sensory nerve activity (NA).
(A) Example trace of an experiment where SSA (20 %) were applied for 90 min. Neurogram (top), firing frequency (mid) and intraluminal pressure were recorded throughout the experiment. Application of SSA increases nerve activity acutely and decreases spontaneous NA and distension-induced firing during long-term application

(B1) Response profile of nerve activity during the application of different concentrations of SSA. An initial increase of NA was observed when 5 %, 10% or 20 % SSA were applied whereas inhibition of nerve activity occurred only at higher concentrations. (B2) The early increase was not significantly different between 5-20 % SSA (v/v). The inhibitory response was only observed in experiments with > 10 % SSA.
(C1) During prolonged application (> 45 min), mechanosensitivity of afferent nerves was profoundly reduced when 10 % or 20 % SSA were bath-applied. (C2) Inhibition of both components of mechanosensitivity (LT, low threshold; HT, high threshold) contributed to the reduction of the distension response at late time points (dist 7, 90 min application) whereas only the high threshold component was significantly reduced at earlier time points.
N (5 % SSA) = 11, N (10 % SSA) = 18, N (20 % SSA) = 14, N (vehicle) = 16

The responses to bath-applied SSA displayed a large inter-individual variability. In the context of *S. aureus* infection, it is established that colonisation with commensal *S. aureus* increases the risk of infection and therefore, we investigated whether intestinal carriage affected the response profile to bath-applied SSA. Consistent with previous studies (Holtfreter et al., 2013; Schulz et al., 2017), we found that ileal and colonic washes contained cultures that proliferated on staphylococcus-selective Baird Parker plates (Fig S2A). The absolute amount of CFU differed between individual mice (Fig S2A) and was also different between ileum and colon with no indication of a consistently higher load in either of these tissues (not shown). When we correlated CFU number and afferent excitation or inhibition, we found a significant inverse relationship between bacterial load in the ileum and nerve activity at 30 min of SSA application (not shown). In agreement with this, the SSA-induced excitation at 30 min was significantly higher in animals with a low compared to animals with high CFU. The inhibitory response was not affected by bacterial load. This suggests that the composition of the host microbiome may contribute to variability in the afferent sensitivity to bacterial mediators.

The numbers of commensal bacteria increase along the length of the intestine which contributes to colonisation resistance and protection against pathogens. Therefore, we investigated whether the response of colon-innervating nerves to SSA differed from small intestinal afferents (Fig S3). The biphasic response consisting of an excitatory and inhibitory phase was also observed in colonic afferents. The median of the excitation at 15 min of application was lower in the colon (128.5 % of baseline, N=8) than in the small intestine but this comparison should be interpreted carefully because vehicle reduced spontaneous nerve activity (NA) in the colon (82.3 % of baseline, $p = 0.03$ compared to SSA). The inhibitory effect was exaggerated. We observed a complete silencing of spontaneous firing in almost all experiments by 60 min of SSA application which was earlier than in in the small intestine (Fig 1). These data suggest that the presence and composition of microbiota may contribute to afferent sensitivity to bacterial mediators but it is possible that factors such as the lower spontaneous discharge in colonic afferents also account for these differences.

Inhibition of mechanosensitivity

Modulation of spontaneous discharge of intestinal afferent nerves (gut-brain axis) may be involved in physiological reflexes and sensations such as fullness after a meal or abdominal discomfort. In contrast, pain sensation is thought to be mediated by the activation of high-threshold mechanosensitive fibers. We investigated the effect of SSA on those fibers by distending the tissue in the presence of SSA.

Ramp distensions were performed every 15 min throughout the experiment and increased nerve activity as a result of the activation of low, wide dynamic range and high threshold fibers as described previously (Rong et al., 2004). Under baseline conditions before SSA application, we observed a 3.1-fold (median) increase of nerve activity in response to distension (Fig 1A). As can be seen in Fig 1A, application of 20 % SSA increased the maximum of the distension-induced firing in parallel with the increase of spontaneous discharge at the 1st SSA distension. At later time points (2nd and 3rd SSA distension), the firing at 30 mmHg remained constant although spontaneous firing increased further. Subsequently, mechanosensitivity was profoundly inhibited during SSA but not vehicle application (Fig S1). In fact, distension did hardly induce any increase of nerve activity at the 6th distension in the presence of SSA (Fig 1C). When we analysed the change of nerve activity at 15 mmHg and 30 mmHg separately to investigate the low threshold and high threshold components, we found that both were reduced simultaneously (Fig 1C2). Consistent with the effects of SSA on spontaneous firing, reduction of SSA concentration to 5 % but not 10 % reduced the inhibitory effect of SSA on mechanosensitivity. This indicates that SSA do not target distinct subpopulations of mechanosensitive or insensitive fibers.

3.2 Intraluminal application of supernatants from *Staphylococcus aureus* cultures increases intestinal afferent nerve activity

Bath application enables a preferential interaction of soluble mediators with serosal and mesenteric fibers which innervate the outer layers of the gastrointestinal wall and are implicated in pain sensation. On the contrary, terminals of mucosal fibers which are considered as sensors of "physiological" stimuli with lower intensity, can be found in the mucosal and submucosal layer of the intestine (Brierley et al., 2004). A possible interaction of those fibers with bacterial compounds can be best studied using intraluminal perfusion.

Spontaneous firing

Soluble mediators in SSA also affected nerve activity when they were perfused through the intestinal lumen (Fig 2). Intraluminal application of undiluted SSA increased spontaneous firing to 150 % of nerve activity compared to the perfusion of Krebs buffer at the beginning of the experiment (Fig 2A). This was significantly higher than the excitation induced by intraluminal perfusion of 100 % vehicle but smaller than the excitation caused by 20 % bath-applied SSA at the 15 min time point (Fig 2B, left). During prolonged intraluminal application, nerve activity tended to increase further which was in sharp contrast to the remarkable inhibitory effect of 20 % SSA during bath application (Fig 2B, right).

Because we observed a smaller degree of excitation and no inhibition during intraluminal application, we hypothesised that the mucosal epithelium acted as a barrier to excitatory and inhibitory mediators. In *ex-vivo* preparations, the mucosal barrier deteriorates over time (Yissachar et al., 2017) and therefore, we expected that application of SSA at a later time point may renewal an inhibitory effect.

When intraluminal application of undiluted SSA was delayed by 90 minutes during which tissue was continuously distended, the response profile was indeed altered compared to early application (Fig 2C). The excitation at 30 min was significantly smaller during late compared to early application ($median_{late}$ = 129.2 % of baseline, $median_{early}$ = 154.6 %, p = 0.029, Mann Whitney test) and late application also revealed an inhibitory effect which was significantly different from the sustained excitation during early application (Fig 2D). The overall response pattern was reminiscent to that of bath-applied SSA albeit with much higher concentrations of SSA mediators and this suggests that the epithelium contributes to the remarkable difference between intraluminal and bath application.

Mechanosensitivity

We also investigated the effect of intraluminal SSA application on mechanosensitivity of small intestinal afferent nerves. Early application had small effects on distension-induced firing. Only at late time points, mechanosensitivity was different between SSA and vehicle at low pressures (Fig 2E, top). Indeed, the low (LT, 15 mmHg) but not the high threshold component (HT, 30 mmHg) was significantly reduced at 90 min (Fig 2E, bottom). Consistent with the epithelium preventing the activity of inhibitory mediators, the decrease of the LT was apparent at earlier time points during late application of SSA and in addition, the maximal increase of distension-induced firing (high threshold) was also significantly reduced during intraluminal SSA_{late} (Fig 2E, bottom). This inhibition of both populations was consistent with our findings from the bath application (Fig 1C). Thus, together with

the findings on spontaneous discharge, it appears that the epithelium is particularly important to protect afferent nerves from inhibitory compounds.

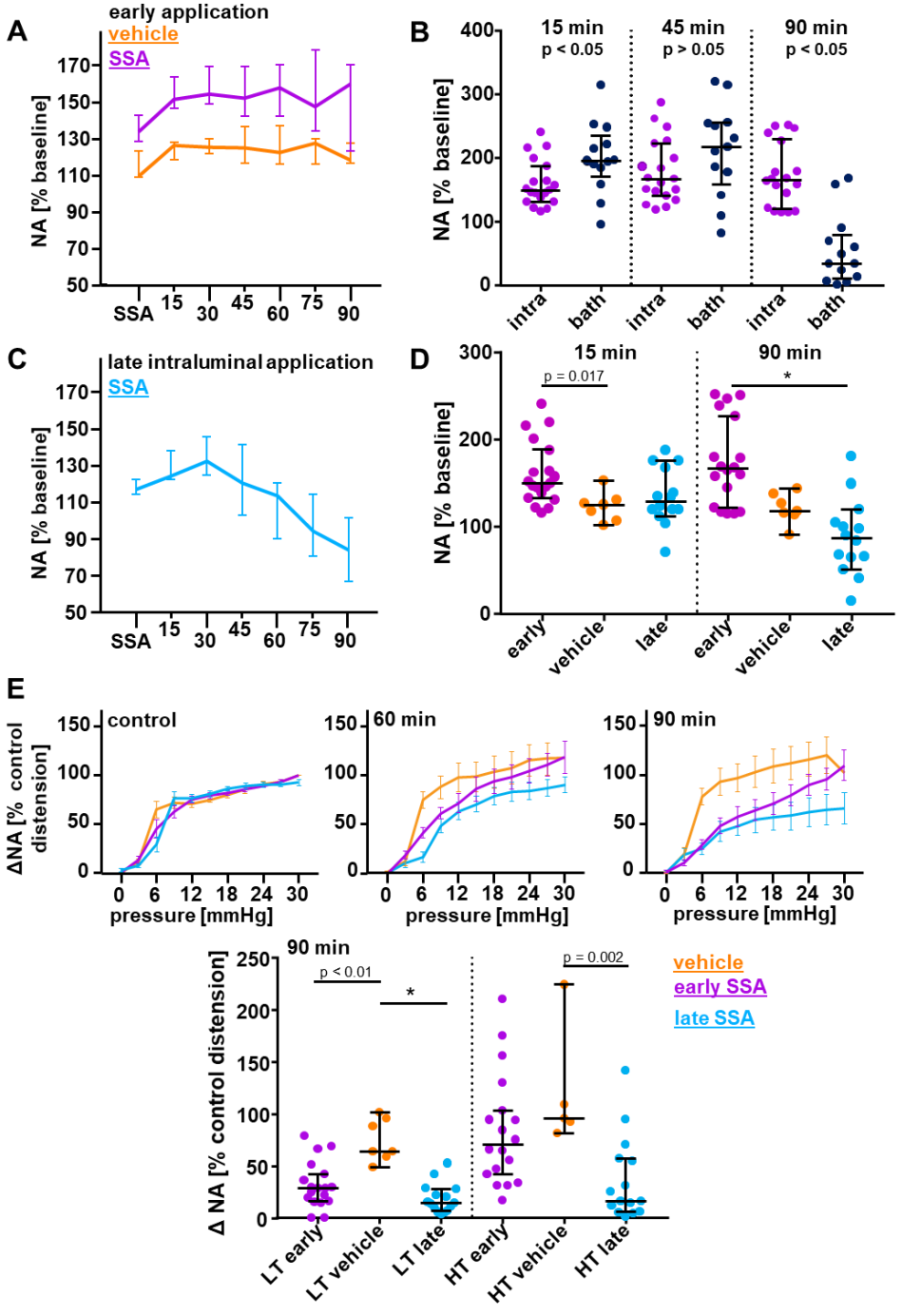


Figure 2: Intraluminal application of SSA (100 % v/v) increases intestinal sensory nerve activity.

(A) Response profile of intestinal afferent activity during the perfusion of 100 % SSA or vehicle through the intestinal lumen. (B) Comparison of the effects of luminally (100 %) and bath-applied SSA on nerve activity. Bath-application induced a larger increase of NA and inhibited NA at 90 min application whereas intraluminal SSA induced a sustained excitation. (C) Response profile of NA during intraluminal application of SSA after 10 distensions under control conditions (perfusion of physiological Krebs) resembled bath application. (D) Excitation during late application was not significantly different from vehicle at 15 or 90 min of application. Compared to the early application, NA was significantly reduced. (E) Distension response profiles of nerves during intraluminal SSA application (top). Early and late SSA application significantly reduced the increase of nerve activity during low intraluminal pressures (low threshold fibers, LT) but only late application also significantly reduced the high threshold component (measured at 30 mmHg). N(SSA_{early}) = 19, N(SSA_{late}) = 14, N(vehicle) = 7, N (SSA_{bath}) = 13. Experiments for late application were performed in Adelaide. Animals from Sheffield and Adelaide did respond similarly to SSA.

3.3 SSA application induces secretion in the intestinal epithelium

The profound differences between intraluminal and bath application of SSA and the changes associated with late application suggest that the mucosal epithelium may also respond to bacterial mediators. Recordings of transepithelial ion movements (short circuit currents, SCC) can be used to investigate the effect of stimulus application on the epithelium (Brighton et al., 2015; Joshi et al., 2013). We measured SCC when SSA were applied to the luminal (apical) or serosal (basolateral) chamber.

Serosal application of SSA induces distinct changes of basal SCC

As can be seen in Fig 3A (straight lines), serosal application of 10 % SSA but not vehicle, induced profound changes immediately after and during long term application. The early response consisted of a small decrease of SCC, a very pronounced increase and a subsequent decrease of SCC within about 30 s after application. The peak of the response (maximum SCC) was significantly higher during SSA compared to vehicle (Fig 3 B1, left). SSA also caused a sustained increase of basal SCC while vehicle tended to decrease it (Fig S2A2). The overall change of basal SCC throughout the duration of the experiment (area under the curve, AUC) was significantly different between SSA (JE2) and vehicle (Fig 3B1, right) indicating that mediators in SSA have a strong pro-secretory when present at the basolateral side.

Indeed, the same response patterns were observed when 5 % and 20 % SSA were applied to the serosal chamber (Fig 3C). The maximum of the early response pattern and the AUC were significantly different between 5 % compared to 20 % indicating a concentration-dependent effect. The comparison between the 5 % and 10 % data was not significant (Fig 3C1, left and Fig S2B, left) although the response to 5 % appeared to be delayed compared to 10 % SSA (Fig S2B, right).

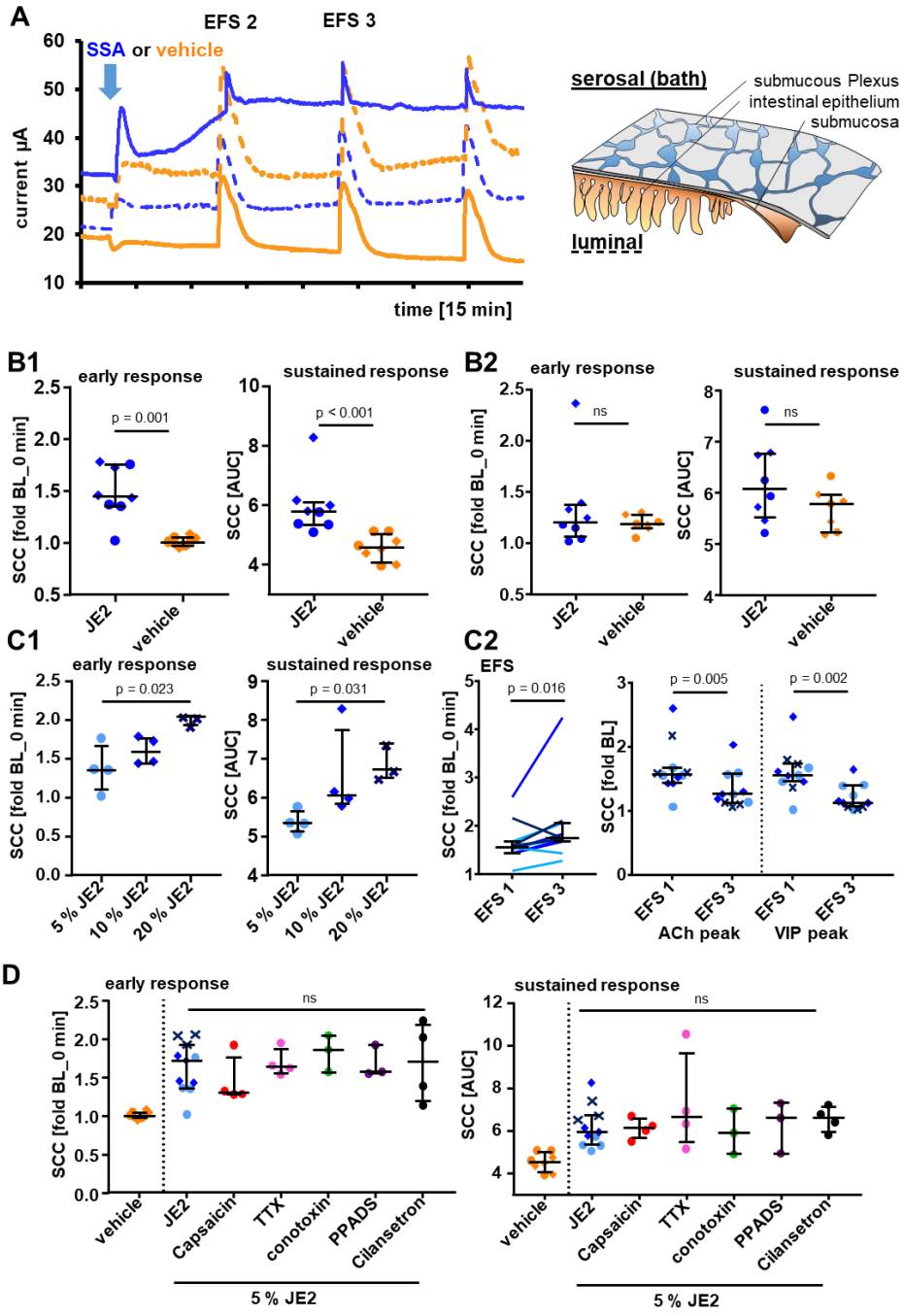


Figure 3: SSA affected short circuit currents in mucosa-submucosa preparations of the guinea pig in Ussing Chambers. (A) Example traces for the serosal (straight lines) or mucosal (dotted lines) application of 10 % SSA or vehicle. The peaks correspond to electrical field stimulation-induced secretion. On the right, a schematic of the mucosa-submucosa preparation is shown. It was prepared by removing the muscle layer with neurons of the myenteric plexus under the dissection microscope. (B1) There was a significant difference between serosally applied SSA and vehicle with regard to the early response pattern and the sustained increase of SCC. AUC was calculated as shown in Fig S2. (B2) There was no difference with regard to either

response pattern when SSA or vehicle were applied to the mucosal side. Data from experiments with 5 % (circles) and 10 % (squares) was pooled as they were not statistically different, performed on tissues from the same animal and equal N numbers. (C1) There were significant different between the maximum during the early response (left) and the sustained response between the application of 5 % and 20 % SSA but not between 5 % and 10% v/v. (C2) Overall, SSA induce an increase of the maximum EFS-induced SCC (left). When EFS-induced secretion was calculated relative to the increased baseline however, the increase of SCC during EFS was smaller during SSA application. This was particularly pronounced for the second (VIP-mediated) phase of EFS-induced secretion. The data for experiments using 20 % SSA was not included in the statistical analysis but is depicted for reference.

(D) Mucosa-submucosa preparations were incubated with blockers of neuronal transmission (ω-conotoxin, tetrodotoxin, capsaicin) and antagonists of serotonergic (cilansetron) and purinergic (PPADS) signaling prior to application of 5 % SSA. When compared to the SSA-induced early (left) and sustained (right) response, none of the blockers significantly reduced either response pattern. Vehicle is depicted for reference but was not included in statistical analysis.

data points per condition were obtained from different animals

Electrical field stimulation (EFS) was used to investigate the effect of SSA on neurogenic secretion and induces a biphasic increase of SCC (Krueger et al., 2010). The ACh peak was higher in the presence of SSA (EFS 3) at any tested concentration (5-20 % v/v) compared to EFS1 whereas vehicle had no effect (Fig 3C2 left, Fig S2A3). However, the increase of EFS-induced secretion was smaller than the increase of baseline SCC indicating a net decrease of EFS-induced secretion (Fig 3C2, right) which could be because the system was maximised. Importantly, neurogenic secretion was not blocked by SSA even at a concentration of 20 %.

Bacterial mediators do not affect short circuit current when applied to the apical membrane

We next tested whether SSA applied to the luminal chamber altered SCC. At a concentration of 10 %, SSA induced a small increase of SCC immediately after application which was maintained until the end of the experiment (Fig 3A, dotted lines). This increase however was also observed during vehicle application indicating that SSA have no additional effect. In agreement, luminal application of both, vehicle and SSA, induced a similar increase of the EFS-induced secretion (Fig S2A3, left). These findings are consistent with a mucosal barrier effect and implies that mediators need to access submucosal targets which include basolateral receptors on the epithelium and the ENS in order to increase SCC.

Secretomotoneurons increase SSC through the release of ACh and VIP but other transmitters such as serotonin and ATP also have prosecretory effects via their respective receptors on epithelial cells. To test the hypothesis that the enteric nervous system is involved in the prosecretory effects of serosal SSA, mucosa-submucosa preparations were pre-incubated with blockers of neuronal activity and signal transduction prior to application of 5 % SSA.

SSA's effects on SCC are not mediated by the ENS

Incubation of mucosa-submucosa preparations with blockers of neuronal activity prior to application of 5 % SSA did not significantly reduce the maximum of the early response pattern (Fig 3D1, left) or the sustained increase of SCC (Fig 3D1, right). We observed no significant differences between SSA-induced changes of SCC when action potential generation was blocked with TTX (Fig 3D, pink) or when synaptic transmission was prevented through blockage of calcium-induced neurotransmitter release with ωconotoxin GVIA (Fig 3D, green). This indicated that modulation of enteric neuron activity does not contribute to the prosecretory effects of SSA. In addition, we also excluded a contribution of extrinsic sensory neurons which express TTX-resistant Na_Vs by pre-incubation with high concentrations of capsaicin (Fig 3D, left, red).

Blocking neuronal activity did not affect the early or sustained response to SSA. *S. aureus* produces proteins with pore-forming and membrane-disrupting capacity and these could potentially lead to transmitter release from neurons but also other resident cells via mechanisms independent of neuronal activity. Because it is known that serotonin and ATP are potent prosecretory signalling molecules, we blocked these pathways using cilansetron (5-HT_3R antagonist) and PPADS (P2X receptor antagonist). Neither of these was able to block SSA-induced changes of SCC (Fig 3D, black and violet) suggesting that serotonin and ATP are not involved in the response patterns.

Together, these findings indicate that SSA increase SCC likely via direct interaction with the basolateral membrane of epithelial cells. The independence of SSA's prosecretory effects from neuronal activity was unexpected given the pronounced changes of intestinal nerve activity. It suggested that extrinsic sensory neurons are more sensitive to components in SSA than intrinsic enteric neurons.

3.4 SSA reduce the amplitude of colonic contractions

Bacterial infections are associated with gastrointestinal dysmotility and therefore, we used a previously validated assay to investigate if SSA can modulate colonic contractions (Accarino et al., 1993; Keating et al., 2010). Distension of colonic segments to an intraluminal pressure of 3-5 mmHg induced colonic migrating motor complexes (MMC) which persisted for at least 2 hours in the presence of vehicle and following washout (Fig 4A). We observed 5 MMC in a time interval of 15 min and this was in agreement with our previous study suggesting that 5 % vehicle does not affect the generation of MMC.

With 5 % v/v SSA the frequency of MMC was unchanged but towards the end of the incubation period and following washout there was a significant decline in contraction amplitude (Fig 4B, right). In addition, the amplitude of cholinergic-mediated contractions was attenuated by SSA perfusion (Fig 4B, right). These findings suggest that soluble mediators released from bacteria affect muscle contractility.

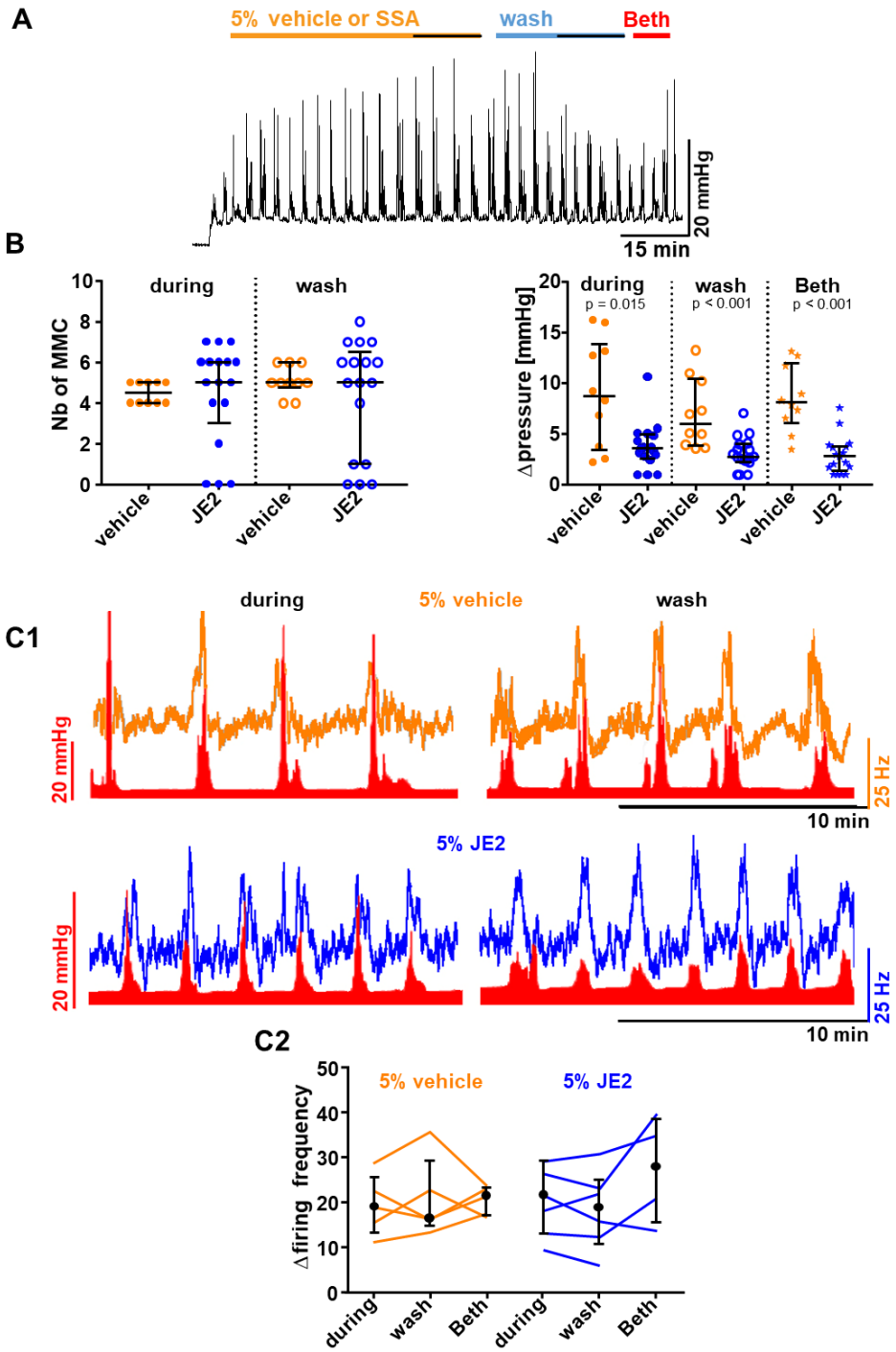


Figure 4: SSA (5 %) altered colonic motility patterns.

(A) Example trace and experimental protocol. SSA (5 % v/v) were bath-applied for one hour after initiation of MMC. Analysis were performed on the 15 min intervals before wash-out and Bethanechol application for the effect of SSA and wash respectively.

(B) Quantification of contractions and maximal amplitude. SSA do not significantly reduce MMC frequency but the amplitude of MMC contractions during SSA and Bethanechol application and the wash period.

N (vehicle) = 10, N (JE2) = 17

(C1) Example traces for simultaneous recordings of proximal colonic afferent nerve activity and contractions. Changes of contractile activity are represented in afferent discharge as periods of high afferent discharge. Vehicle (top) or SSA (bottom) were applied. Changes in pressure are depicted in red and nerve activity in orange (5 % vehicle) and blue (5 % JE2). (C2) SSA did not affect the average change of nerve activity significantly.

N (vehicle) = 5, N (JE2) = 6

Reduction of muscle contractility is not reflected in colonic afferent nerves

The profound decrease of contraction amplitude prompted us to record the activity of sensory nerves innervating the proximal colon during migrating motor complexes because afferents are known to be mechanosensitive. After initiation of MMC, afferent nerves displayed rhythmic intervals of high and low firing frequency that reflected colonic contractile activity (Fig 4C1). This activity was maintained during application of 5 % SSA and in line with our experiments in the small intestine with 5 % SSA, we did not observe a decrease of spontaneous activity. To our surprise however, the distension-/contraction-induced firing was also maintained despite the profound decrease of contraction amplitude (Fig 4C1, bottom). Comparison of the contraction-induced nerve activity between SSA application, wash-out and Bethanechol application shows that it was not different at any of the three time points (Fig 4C2). This suggests that proximal colonic afferent nerves are sensitive to signals encoding the initiation of MMC.

4 Discussion

In this study, we have shown that soluble mediators from *S. aureus* exerted a biphasic, excitatory and delayed inhibitory, effect on the spontaneous discharge of small intestinal afferent nerves. In addition, mechanosensitivity was remarkably reduced when those mediators were applied to the serosal side of the intestine. Serosal application of SSA also increased secretion in Ussing chamber experiments and decreased the amplitude of migrating motor complexes. In contrast, luminal application had a comparatively small excitatory effect on afferent activity and did not increase short circuit currents compared to vehicle.

4.1 SSA have excitatory and inhibitory effects

The profound effects of bath-applied supernatants reminded of the dual activity of TRPV1 agonists (Alsalem et al., 2016). Application of SSA as well as high concentrations of capsaicin or resiniferaoxin induce a profound excitation which is followed by desensitisation of sensory nerves or isolated neurons (Mcmahon et al., 2015). Consistent with capsaicin- or resiniferatoxin-mediated ablation of sensory TRPV1-expressing neurons, we found that SSA completely abolished the afferent response to distension. However, our findings argue against the hypothesis that in the case of SSA, inhibition is a consequence of over-excitation. We found that at a concentration of 5 % v/v, the degree of excitation was not significantly different to 20 % SSA but 5 % SSA did not inhibit nerve activity. In addition, we observed inhibition of nerve activity in experiments where only a small amount of excitation was recorded. This suggests that distinct components of SSA mediate excitation and inhibition of the afferent response.

An interaction between intestinal afferents and bacterial mediators from *S. aureus* has not been previously investigated and therefore, we cannot conclude about the identity of neuromodulators in SSA. Evidence from previous studies investigating *S. aureus* in pain implicate that virulence factors are excitatory. Chiu et al. (2013) showed that α-haemolysin, a pore-forming toxin that lyses immune cells (Becker et al., 2014), and formyl peptides induce calcium transients and action potential firing in dorsal root ganglia neurons (Blake et al., 2018). In addition, staphylococcal enterotoxins mediate emesis during *S. aureus* food poisoning through inducing serotonin release (Hu and Nakane, 2014) which is indicative of an excitatory activity (Hicks et al., 2002). On the contrary, studies with other bacteria indicate that bacterial proteases and lipid mediators may be able to inhibit afferent nerve activity (Pérez-Berezo et al., 2017; Sessenwein et al., 2017). *S. aureus* also produces proteases and lipid

mediators which are involved in its success as a pathogen but it is currently unknown whether those also affect neuronal activity (Chapman et al., 2017a; Strobel et al., 2016). To understand the mechanisms underlying SSA-induced neuromodulation, future studies should aim at identifying the components of SSA that contribute to excitation and inhibition. The inhibitory substances may be particularly interesting because they could potentially be used as a novel approach for pain therapy.

In addition to identifying the mediators that are involved in the neuromodulatory effects of SSA, future studies should also investigate the mechanisms contributing to the changes of intestinal nerve activity. The bacterial mediators that have been investigated to date exert both receptor-dependent and -independent activities. Formylpeptides were shown to induce calcium transients via the formyl-peptide receptor (FPR) whereas the inhibitory effects of proteases and lipopeptides required protease-activated receptor-4 (PAR-4) and the γ-amino butyric acid receptor B ($GABA_B$) respectively (Pérez-Berezo et al., 2017; Rautenberg et al., 2011; Sessenwein et al., 2017). In contrast, it is suggested that α-haemolysin contributes to depolarisation through the formation of calcium-permeable pores (Menestrina, 1986). Pore formation can also lead to cell death and indeed SSA have strong cytotoxic activities against immune cells but not towards neurons (Blake et al., 2018). We perfused undiluted SSA (100 %) through the intestinal lumen and did not observe a decrease of nerve activity or a complete disruption of the tissue which indicates that cytotoxicity is not the primary mechanism underlying nerve inhibition. Furthermore, we found that mucosa-submucosa preparations continued to respond to electrical field stimulation after application of 20 % SSA directly to the neurons and this also argues against a strong neurotoxic effect of SSA.

4.2 Apical bacterial sensing and neuromodulation are not involved SSA-induced secretion

Findings from several studies suggest that epithelial cells can sense bacteria or their metabolites through receptors expressed on the apical surface (Bellono et al., 2017; Cox et al., 2010; Yano et al., 2015). However, a clear separation between apical and basolateral is not possible in in-vitro studies using isolated cells because the epithelial polarisation is disrupted. Our data suggest that epithelial sensing of mediators in SSA engages basolateral mechanisms. In Ussing Chamber experiments, luminally applied supernatants did not increase short circuit current compared to vehicle whereas serosal application induced an immediate response pattern and a sustained increase of SCC. The hypothesis of basolateral sensing is consistent with Ussing chamber experiments in the studies of Kwak et al. (2012) and Brighton

et al. (2015). The PFT α-haemolysin only increased tissue permeability when it was applied to the basolateral chamber (Kwak et al., 2012) and similarly, it was basolateral bile acids that predominantly contributed to Glp-1 secretion from intestinal epithelia (Brighton et al., 2015). With regard to our findings, this suggests that epithelial cells respond to bacterial products predominantly after bacterial translocation or bacteraemia which is also in line with a stronger neuromodulatory effect of bath-applied compared to luminally perfused SSA.

Intestinal secretion can be modulated either through direct interaction with epithelial cells or via secretomotor neurons from the ENS (Thiagarajah et al., 2015). Several of our findings are in favour of a neuron-independent mechanism underlying SSA's prosecretory effect. We found that the maximal EFS-induced secretion was not affected during SSA application even at a concentration of 20 %. In addition, blocking neuronal activation, neurotransmission or serotonin and ATP signalling did not reduce the prosecretory effect of SSA. This suggest a direct interaction of soluble mediators from *S. aureus* with epithelial cells which has been previously reported for other pathogenic bacteria such as *Vibrio cholera* and *Escherichia coli* (Thiagarajah et al., 2015). Given the strong neuromodulatory effect of SSA in the afferent nerve recording experiments however, this finding was unexpected but indicates that ENS neurons may be less susceptible to SSA then sensory neurons. This idea is supported by the results of our motility recording experiments. Here, the contraction amplitude but not the frequency of MMCs was significantly reduced by SSA which is indicative of an altered muscle contractility rather than neuromodulation. Others have suggested that modulation of neuronal activity underlied the inhibitory activity of *Lactobacillus* strains on intestinal motility (Wang et al., 2009, 2010; Wu et al., 2013). Thus, pathogens and commensal bacteria may employ different mechanisms to modulate intestinal motility.

4.3 Afferent nerve recordings from the proximal colon

In this study, we have also described a recording technique that allows to investigate the relationship between physiological contractions in the proximal colon and mechanosensitivity of the innervating afferent nerve. We have shown that the contraction/distension-induced increase of afferent discharge was maintained despite a decrease of contraction amplitude which indicates that physiologically generated distensions (i.e. MMC) induce an "all-or-nothing" response in afferent neurons. Although this appears to contradict previous studies that have shown an intensity coding of colonic afferents, it should be noted that our recordings constitute the integrated response of afferent nerves to muscle

contractions, ENS activity that generates MMC and bacterial supernatants. This is in contrast to experiments in the distal colon that have investigated mechanosensitivity using ramp distension or stimulus application to receptive fields (Brierley et al., 2004; Nullens et al., 2016). Therefore, to better understand the relationship between MMC and afferents, future studies should be performed with drugs that have well-defined effects on MMC, ENS and afferent activity.

4.4 Physiological interpretation

We have shown that bacterial mediators modulate the activity of extrinsic neurons that are implicated to contribute to abdominal pain, discomfort, food intake, behaviour, nausea and vomiting. Several studies support the hypothesis that this direct neuronal activation is important during infection. Chiu et al. (2013) and Meseguer et al. (2014) have found that skin infection with *S. aureus* and injection of LPS induce an inflammation-independent spontaneous pain responses (Chiu et al., 2013; Meseguer et al., 2014). Furthermore, physical or chemical ablation of vagal fibers has been shown to reduce sickness behaviour and alterations of food intake (Bluthé et al., 1994; Cawthon and de La Serre, 2018a; Rao et al., 2017b). Excitation might also contribute to the successful eradication of the pathogen by activating the immune system (Mcmahon et al., 2015). Consistent with this hypothesis, we found a higher degree of excitation in animals with lower abundance of endogenous *Staphylococci.* In this regard, the profound inhibition that we have observed during bath application could be interpreted as a mechanism by which *S. aureus* prevents this neuronally mediated inflammation. Alternatively, through inhibiting intestinal sensory pathways that signal to hunger and satiety control centres in the brain, *S. aureus* may modulate food intake for its own benefit. However, further investigations are required to fully elucidate the physiological function of neuronal modulation in the context of infection.

In summary, the main findings of the present study are that bacterial mediators have a dual effect on afferent firing and also influence motility and secretion when they are present at the serosal side. The absence of luminal effects suggests that the epithelium is able to buffer or attenuate these mediators implying that bacterial translocation may be necessary to evoke a response. These effects may precede immune activation and contribute to a coordinated neuro-immune response that serves to contain the pathogenic organism.

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6 Supplementary Figures

Fig S1

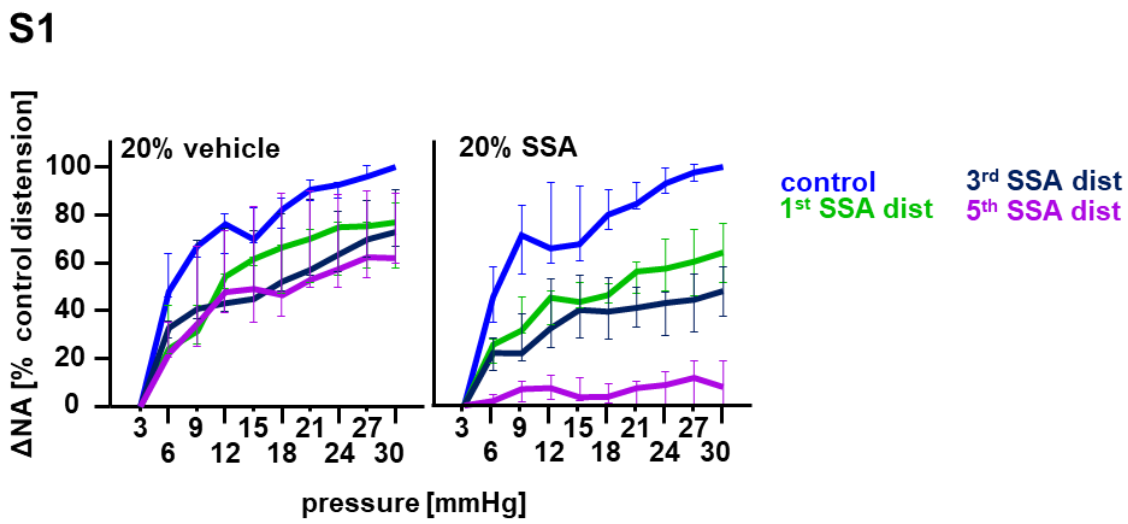

Figure 5(S1): SSA (20 % v/v bath) induce profound changes of mechanosensitivity. Vehicle-induced (left) and SSA-induced (right) changes of mechanosensitivity over time. Distension responses are normalised to the peak firing at (30 mmHg) of the control distension. SSA induce a profound decrease of distension-induced firing. N (JE2) = 14, N (vehicle) = 16

Fig S2

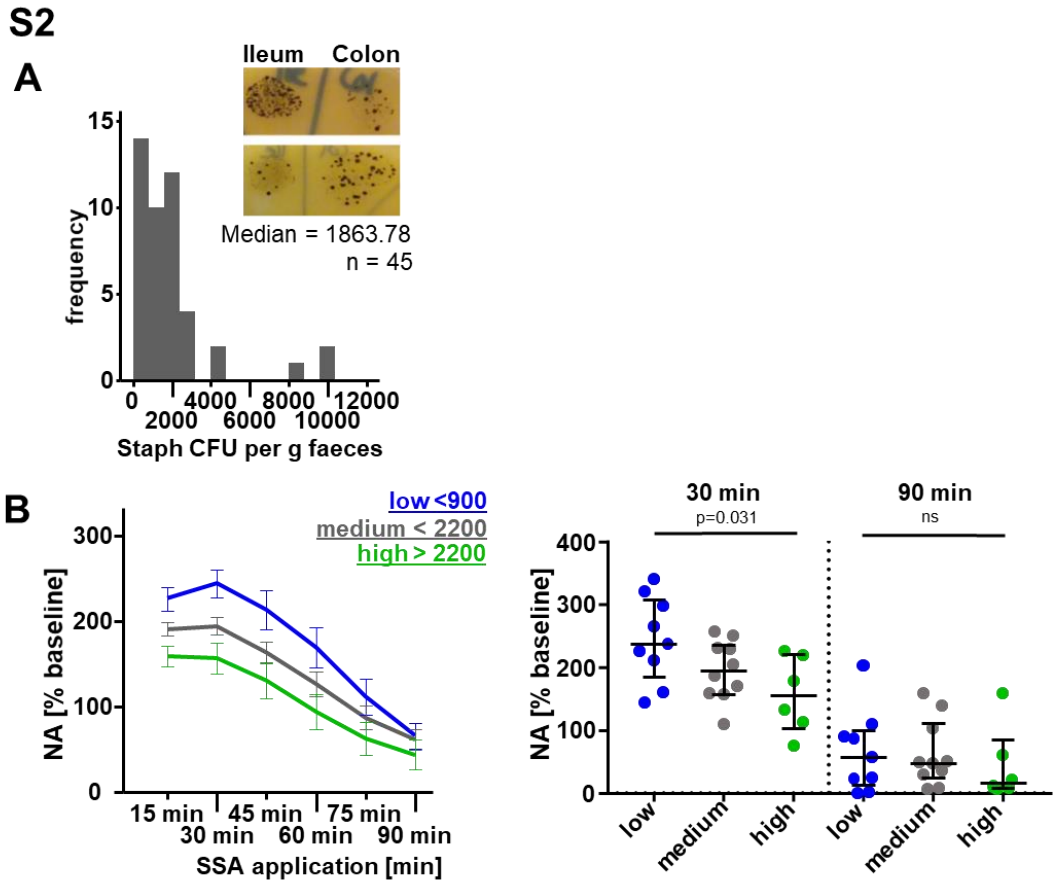

Figure 6(S2): The bacterial load affects the excitation induced by SSA.

(A) Tellurite-containing Baird Parker Plates were used to determine intestinal colonisation with endogenous Staphylococci. The amount of bacteria in murine intestines differed between animals. In some, bacterial load was higher in the ileum whereas in others, the colon contained more endogenous Staphylococci.

(B) Comparison of the response profile for mice with low, medium of high Staph load (left). There was a trend towards an increased excitatory effect of SSA in animals with low numbers of Staph in the ileum compared to animals with large numbers of Staph CFU. The amount of Staph CFU did not impact the inhibitory effect of SSA.

N (low) = 9, N (medium) = 10, N (high) = 6

Fig S3

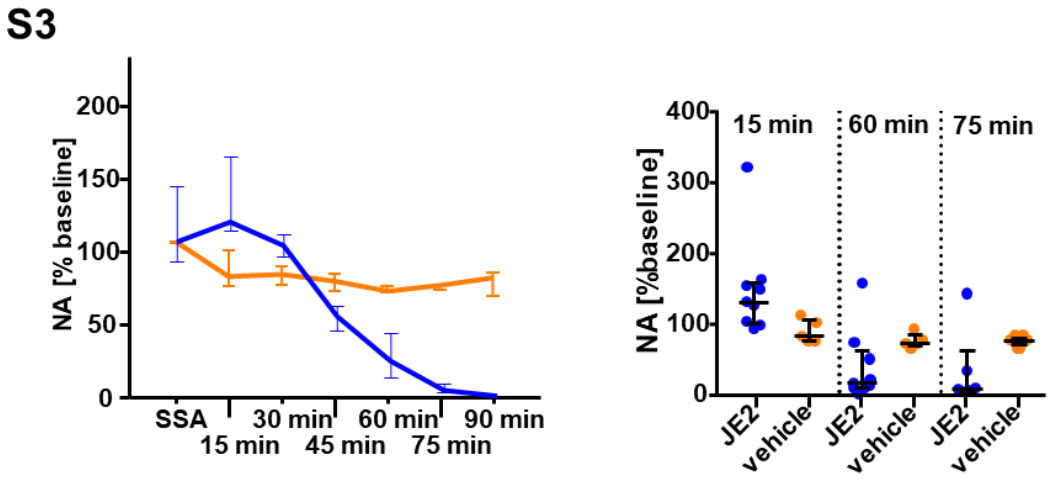

Figure 7(S3): Bath-applied SSA (20 %) induce a biphasic response in colonic afferents.

(left): Response profiles of colonic afferent nerves to SSA (blue) and vehicle (orange) was different. SSA increased spontaneous discharge at early time points. This increase started to reverse after 30 min and nerve activity decreased further until the end of the experiment. (right): SSA induced a significant excitation compared to vehicle at 15 min and inhibited nerve activity at 75 min of application whereas vehicle did not.

Non-parametric statistical tests (Mann-Whitney test); N(JE2) = 8, N(vehicle) = 4

Fig S4

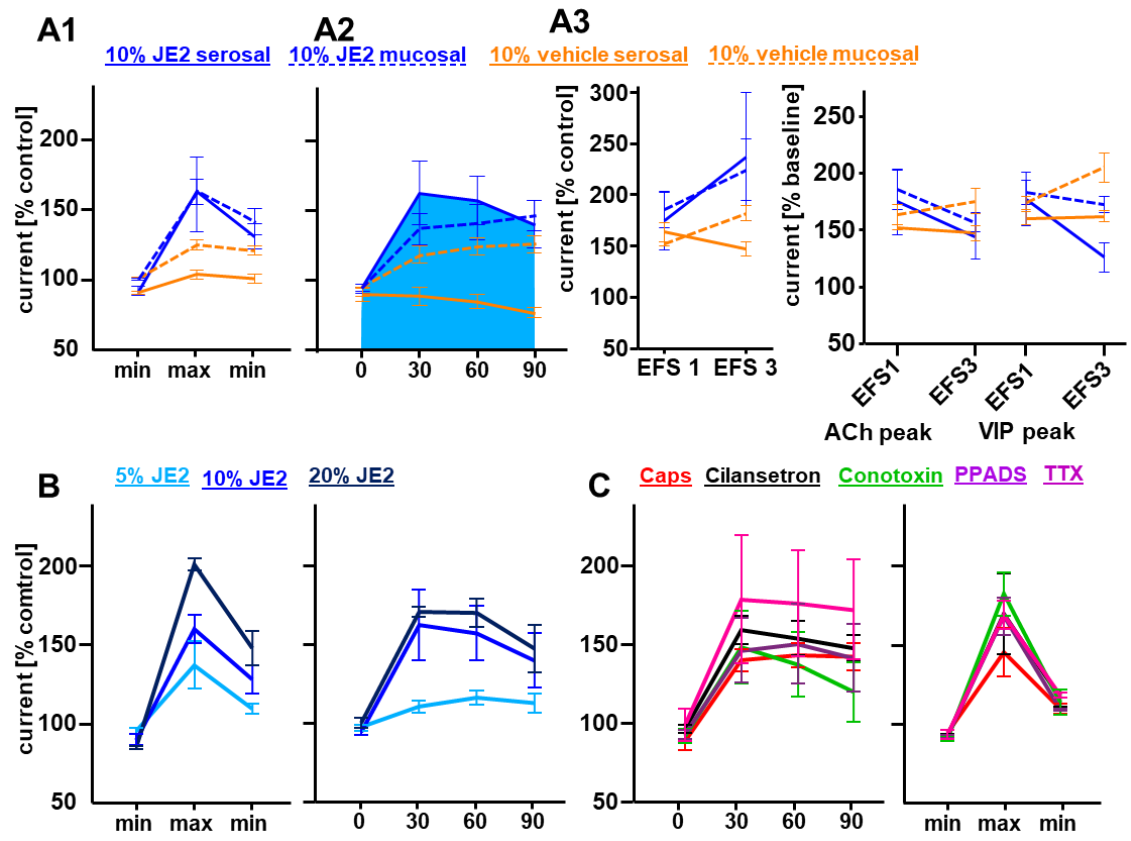

Figure 8(S4): SSA affect short circuit currents (SCC) independent of neuronal mechanisms.

(A) Comparison of the SSA-induced changes of short circuit currents between mucosal (dotted lines) and serosal (straight lines) for a concentration of 10 % SSA. (A1) Serosal application of SSA but not vehicle increased SCC immediately after application. (A2) In addition, a slower sustained increase was observed. The overall increase (AUC) has been calculated as sum of SSCs at every time point. (A3) There was a significant increase of EFS-induced maximal secretion during SSA application which was not observed during vehicle application (left). If the increase of baseline SSC was taken into account, the EFS-induced increase of SCC was reduced during SSA application whereas vehicle has no effect. Neither the early response (A2) nor the sustained increase of baseline SCC or EFS (A3) were affected differently when vehicle (orange) and SSA (blue) were applied to the luminal side (dotted lines).

(B) Concentration-dependent effect of SSA on early response (left) and sustained response (right). Both responses were more pronounced at higher concentrations.

(D) Pre-incubation of mucosa-submucosa with blockers of neuronal transmission and antagonists of serotonergic and purinergic signaling did not affect the early response pattern. The sustained response tended to be higher than during 5 % SSA application.

For statistical analysis see Fig 3, N = 3-4 for each data point

CHAPTER III

Small intestinal afferent sensitivity and sensing of *Staphylococcus aureus* after TNBS colitis

***ABSTRACT*:**

Chronic visceral pain is a major clinical health problem that often develops after gastrointestinal infection and inflammation. Peripheral sensitisation of extrinsic nerves through cytokines that are released during the active disease is implicated in disease pathogenesis. Chemically induced colitis has been used to study visceral pain in animal models. It causes hypersensitivity of colonic pelvic and splanchnic afferents but it is currently unknown to what extent other peripheral nerves are affected. The effect of inflammation on the afferent response to bacterial products has also not been investigated. Electrophysiological recordings from afferent nerves were performed in an *ex-vivo* preparation of the distal small intestine from healthy adult male C57BL/6 mice and animals that received TNBS (2,4,6-Trinitro-benzenesulfonic acid) via colonic instillation 21-28 d before the experiment. Spontaneous discharge and the response to distension were recorded under control conditions (physiological Krebs buffer) and in the presence of supernatants from cultures of *Staphylococcus aureus* (SSA) which were either bath-applied or perfused through the intestinal lumen.

In mice from two different colonies (in-house breeding colony and the Jackson laboratory), small intestinal afferents did not display mechanical hypersensitivity 21-28 days after TNBS-colitis. In healthy and TNBS-treated mice, SSA application to the bath induced a biphasic response consisting of an initial excitation and a profound inhibition of spontaneous discharge, and a reduction of mechanosensitivity. The extent of inhibition was reduced in a subset of animals from the Jackson laboratory. Intraluminal perfusion increased spontaneous discharge and did not affect mechanosensitivity in either of the animals. However, intraluminal application of SSA started to inhibit nerve activity and the response to distension when they were applied with a delay of 90 min in healthy but not TNBS-treated animals.

These results suggest that colitis-induced hypersensitivity is limited to neurons innervating the site of inflammation. They also indicate that colitis causes adaptive changes in the small intestine that reduce the inhibitory activity of SSA.

1 Introduction

Functional gastrointestinal diseases including dyspepsia, idiopathic chronic constipation and irritable bowel syndrome (IBS) affect more than 10 % of the general population. Patient's quality of life is severely impaired by chronic abdominal pain and an altered stool frequency/consistency (Quigley et al., 2006). These symptoms are also used for the diagnosis and classification of patients into 4 subtypes of IBS: diarrhoea predominant (IBS-D), constipation predominant (IBS-C), mixed bowel habits (IBS-M) or unclassified IBS (Lacy and Patel, 2017). Despite much effort, the pathogenesis of IBS is yet to be fully uncovered. However, both central and peripheral mechanisms are implicated (Valdez-Morales et al., 2013b; Verne et al., 2012).

The term gut-brain axis is often used to describe the sensory pathways connecting the gut and the brain and cell bodies of intestine-innervating sensory nerves can be found in the nodose ganglia (NG), thoracolumbar (T9-L2) and lumbosacral (L6-S1) dorsal root ganglia (DRG) (Furness et al., 2013). These three populations of sensory neurons make different contributions to the innervation along the length of the intestine and also convey different information. The small intestine is densely innervated by vagal fibres that are activated by physiological stimuli. In contrast, spinal splanchnic and pelvic pathways dominate the innervation of the colorectum. Splanchnic afferents have been shown to respond to high threshold/nociceptive stimuli whereas vagal and pelvic nerves are also activated by mechanical stimuli with lower intensity, mucosal stroking and stretch (Brierley et al., 2004). In addition to this mechanosensitivity, a number of endogenous mediators have been shown to activate and modulate afferent sensitivity especially subsequent to inflammation. More recently, cytokines released by the immune system and bacterial mediators have also been shown to alter afferent firing and mechanosensitivity (Campaniello et al., 2017; Ochoa-Cortes et al., 2010).

Hypersensitivity of sensory pathways is considered a hallmark of IBS. Studying visceral hypersensitivity and chronic abdominal pain remains a challenge. The lack of a clear pathogenesis impedes the development of appropriate animal models. Most models have focused on inflammation and injury but psychological stress is also a factor. Chemicals such as TNBS, DSS and oxazolone have frequently been used to induce inflammation in mice, rats and guinea pigs (Moore et al., 2002; Shah et al., 2016; Wirtz et al., 2017). Visceral hypersensitivity develops acutely after establishment of colitis and can persist when the inflammation has resolved (Hughes et al., 2009). This long-term sensitisation of colonic afferents has been observed after colonic TNBS instillation and affects all levels of the sensory pathway (Brierley and Linden, 2014). Increased muscle responses to colorectal distension, nerve activity

of intestinal afferents, excitability of dorsal root ganglia neurons and pErk expression of spinal cord secondary neurons have been described in animals up to 21-28 d after TNBS treatment. After colonic instillation, TNBS-induced hypersensitivity is not restricted to colonic visceral afferents. Instead, hypersensitivity has also been reported for urinary bladder sensory nerves and somatic sensory nerves (Grundy and Brierley, 2018). The mechanisms underlying this cross-organ sensitisation are still incompletely understood but it is suggested that it is linked to the close proximity of sensory neurons innervating different peripheral organs in dorsal root ganglia. Depending on the experimental protocol, the composition of the endogenous microbiota, the presence of environmental bacteria, food composition and the genetics as well as the immunocompetency of the strain of the mice variable levels of colonic and cross-organ hypersensitivity have been observed.

It is also becoming increasingly clear that the small intestine may play an important role in the pathogenesis of IBS which can be considered a pan-gastrointestinal syndrome. However, the extent to which colonic inflammation can lead to altered small intestinal sensory signalling has not been investigated and was one aim of this study. In addition, the small intestine houses less commensal bacteria which may make this region more vulnerable to colonisation with pathogenic bacteria. Indeed, small intestinal bacterial overgrowth is frequently found in IBS patients and may contribute to symptoms (Chang and Lin, 2016). Thus, a second aim of this study was to determine if supernatant from a pathogenic strain of *S. aureus* has a differential effect on small intestinal afferent sensitivity in a post-inflammatory model of colitis. We have previously shown (Uhlig, this thesis) that virulence factors from SA have profound effects on small intestinal afferent firing. In the current study, we determined the extent to which this sensitivity is altered in a post-inflammatory model of TNBS-colitis. We hypothesised that sensitivity would be increased but found this not to be the case and indeed observed a protection from the inhibitory effect of bacterial supernatant following colitis.

2 Methods

Mice and TNBS treatment

All experiments in this study have been performed in accordance with regulations set in place at the South Australian Health and Medical Research Institute (SAHMRI) and were approved by the local Animal Ethics Committee. Mice were obtained from the in-house breeding colony of C57Bl/6 mice (SAHMRI animals) or directly from the Jackson laboratory (Jax animals). The latter were housed separately from the SAHMRI animals and staff were not allowed to handle these mice after contact

with SAHMRI animals on the same day. All mice were objected to a 12/12 hours light cycle and obtained a standard rodent diet. Experiments were performed on adult (> 10 weeks) male animals.

To induce colitis, otherwise healthy 20-30 g C56Bl/6 animals from either colony were administered with 0.1 mL 2,4,6-Trinitrobenzenesulfonic acid (TNBS) (135 µL/mL of 1M solution in 35% ethanol) via colonic instillation as described previously at 13 weeks of age (Harrington et al., 2012; Hughes et al., 2009). Animals were lightly anaesthetised with isoflurane (2-4 % in oxygen) to allow anal insertion of a polyethylene catheter 3 cm into the colon. TNBS-treated animals were housed individually and monitored twice daily for changes in body weight, physical appearance and behaviour until their colitis symptoms had resolved (day 7). Small intestinal nerve recording experiments were performed on 21-28 d after TNBS treatment following euthanasia (rising CO_2 inhalation and exsanguination) according to institutional guidelines.

Cultivation of* S. aureus *and supernatant preparation

S. aureus supernatants (SSA) were produced as previously described (supplementary methods, p 198). *S. aureus* USA300 (JE2) was plated onto sterile tryptic soy broth (TSB) agar plates (1.5 %) and incubated at 37 °C overnight. A 250 mL flask with 50 mL sterile TSB was inoculated with a single colony of *S. aureus* and grown overnight at 37 °C on a rotary shaker. Optical density was determined and suspension culture diluted into 500 mL TSB (2 L) to and OD_{600} of about 0.15. This was incubated for another 24 hours at 37 °C on a rotary shaker after which OD_{600} was determined to confirm bacterial growth. Suspension culture was centrifuged (5.000 rpm, 15 min), aliquoted and stored at -20 °C until usage. On the day of experiment aliquots were defrosted in a warm water bath (37 °C) and diluted to 20 % by volume in Krebs buffer ready for either bath application or luminal perfusion at a rate of 100 µL/min.

Electrophysiology

Whole nerve recordings from small intestinal afferents were performed as described in detail in the supplementary methods (Chapter VI, p 195). The intestine was removed from the animal after euthanasia and placed into fresh carbogenated Krebs solution (mM: NaCl 120, KCl 5.9, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 15.4, glucose 11.5, and $CaCl_2$ 1.2). Using a light microscope, segments from the distal small intestine proximal to the ileocaecal valve with attached mesenteric arcades were dissected and placed into an organ bath chamber. The proximal and distal end of the segment were tied on to an intraluminal perfusion system and the bath was constantly perfused with fresh Krebs

solution (5 mL/min). The nerve was dissected and placed into an electrode to record its activity. Distensions were performed by closing the outflow tab at the anal end of intestine. Electrical signals were amplified and filtered using the NeuroLog headstage and acquired by Micro 1401 running Spike2 software (CED Cambridge).

Following stabilisation (60 min), tissue was continuously distended to 30 mmHg every 15 min. Experimental protocols were started after two reproducible distensions. Bacterial growth medium (vehicle) was bath-applied for 15 min (one distension) prior to bath application of 20 % SSA for 90 min (six distensions). For intraluminal application, undiluted SSA were perfused for 90 min either immediately after stabilisation (early application) or following 10 control distensions (late application).

Data analysis and statistics

Individual experiments were analysed offline (Spike2) and data combined and further processed using MicrosoftExcel® (supplementary methods, p 195). Thereafter, for mechanosensitivity, area under the curve was calculated by addition of the 10 individual values (3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 mmHg). The overall excitatory and inhibitory activity of bath-applied SSA are expressed as area und the curve (AUC) and were determined by addition of %baseline values between 0-45 min and 60-90 min respectively. Statistical analysis was performed using SPSS statistics (version 23, IBM) and GraphPad Prism® (version 7). Non-parametric statistical tests were used and data is presented as median with interquartile ranges.

3 Results

3.1 Colonic TNBS treatment does not sensitise small intestinal afferents

Afferent nerves from TNBS-treated animals and healthy control (HC) displayed spontaneous activity and responded to ramp distension (Fig 1A1). In time-control experiments, we observed a small, not significant, rise of spontaneous firing in HC control mice. Distension activates low, wide dynamic range and high threshold fibers which results in a biphasic increase of whole nerve discharge (Fig 1B). The response to distension was maintained over time but when normalised to spontaneous discharge showed a non-significant decline over time (Fig 1B1). Surprisingly, and despite a clearly developed colitis during the first week after treatment, spontaneous nerve activity (NA) was lower in TNBS animals compared to HC from the in-house breeding C57Bl/6J colony (SAHMRI, Fig 1A2). As in HCs,

spontaneous discharge increased non-significantly in time control experiments (not shown). Again to our surprise, the overall response pattern to distension over time (Fig 1B1) as well as the area under the curve (shown for the first distension, Fig 1B2) were not significantly different between HC and TNBS animals from the SHAMRI colony.

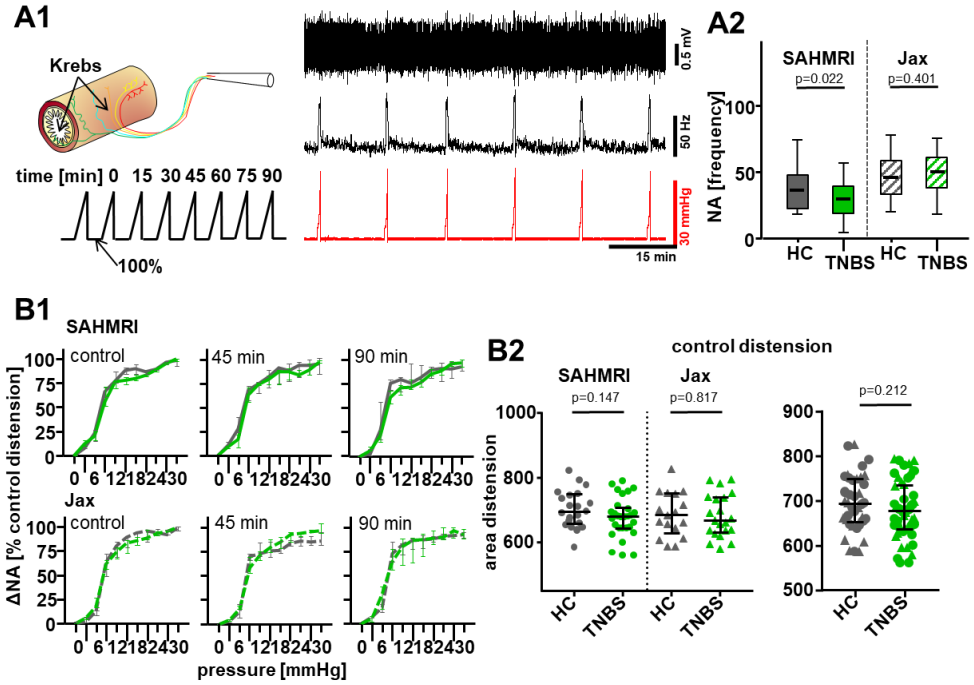

Figure 1: Effect of colonic TNBS treatment on firing characteristics of small intestinal afferent nerves under control conditions. (A1) Example (right) of a small intestinal afferent recording from a TNBS-treated animal where physiological Krebs solution was perfused through the lumen and bath (control conditions) and tissue was distended every 15 min. Traces represent afferent discharge (top), firing frequency (middle) and pressure channel (bottom). (A2) Baseline firing frequency at the beginning of the experiment differs between animals from different origins and was decreased after TNBS treatment in SAHMRI animals. (B1) TNBS treatment did not affect mechanosensitivity in mice from two different origins. (B2) Quantification of the distension response at time point "0" as area under the curve does not reveal differences between healthy and TNBS-treated animals.

N (SAHMRI, HC) = 8, N (SAHMRI, TNBS) = 14, N (Jax, HC) = 6, N (Jax, TNBS) = 5, N (B) includes data from the first distensions of experiments were nerves were stimulated afterwards (Fig 2, 3)

The absence of TNBS-induced small intestinal hypersensitivity prompted us to repeat these experiments in the same strain of mouse from a different supplier (JAX) since environmental factors such as the microbiome may impact on afferent sensitivity. Spontaneous discharge in these mice tended to be higher than in SAHMRI animals and TNBS treatment did not reduce the frequency of action potentials (Fig 1A2). Consistent with the SAHMRI mice however, the level of spontaneous discharge and the response to distension were not augmented in the TNBS-treated JAX mice (Fig 1A3). Thus,

this data does not support the hypothesis that TNBS-colitis causes a profound changes of small intestinal afferent sensitivity.

3.2 The response to bacterial mediators is partially altered in TNBS-treated animals

TNBS treatment is a well validated model of mucosal injury and inflammation leading to increased mucosal permeability and bacterial translocation (Campaniello et al., 2016; Wirtz et al., 2017). During infection, bacteria release virulence factors that enable tissue invasion and we have previously shown that these mediators secreted during growth of the gram-positive bacterium *S. aureus* modulate small intestinal afferent activity. Therefore, we hypothesised that TNBS treatment might affect the response of small intestinal afferents to supernatants from *S. aureus* cultures (SSA).

Bath application

As described previously, bath application of 20 % SSA (v/v) had profound effects on small intestinal nerve activity (Fig 2A1). The response was biphasic with an increase in spontaneous afferent discharge between 0 and 45 min of application which reversed to profound inhibition during prolonged application (Fig 2A2). There was no significant difference in the magnitude and profile of the response when comparing HC to TNBS treatment in the SAHMRI mice (Fig 2A2 top and Fig 2A3). However, the magnitude of the inhibitory response to SSA was significantly attenuated in the JAX mice treated with TNBS compared to HC (Fig 2A3, bottom and Fig 2A3).

The mechanosensitivity of small intestinal afferent nerves during ramp distension was also attenuated by SSA. Compared to the control distension ("0 min", Fig. 2A1), the distension-induced increase of nerve activity was reduced in both HC and TNBS-treated animals from the SAHMRI and the JAX facility after SSA (Fig 2B1, S2). The degree of inhibition at the end of the experiment was similar between TNBS-treated and healthy animals. In both groups, mechanosensitivity was almost completely abrogated (Fig. 2B1, 75 min). However, the onset of inhibition however was significantly delayed in TNBS animals. When the distension response was compared after 15 min exposure to SSA, the degree of inhibition was significantly attenuated in TNBS animals. Together with the effects on spontaneous firing, these results suggest that the TNBS-induced inflammatory insult results in subtle changes of afferent nerve sensitivity to bath-applied SSA but the overall response pattern was not affected by TNBS treatment or the source of the mice.

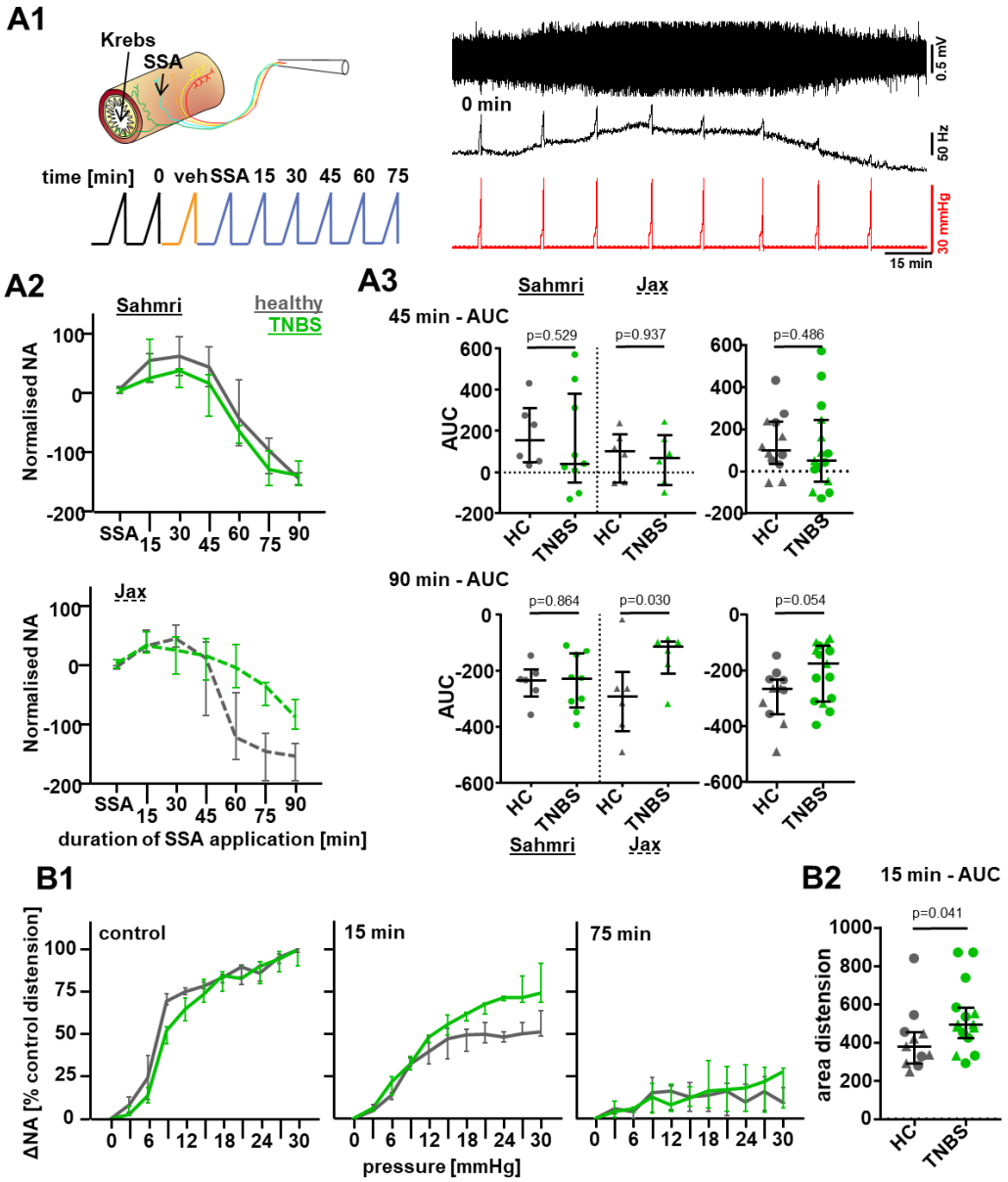

Figure 2: Effect of colonic TNBS treatment small intestinal nerve response to bath-applied supernatants from S. aureus (SSA). (A1) Schematic representation of experimental conditions. Vehicle (veh) and SSA (20 % v/v) were bath-applied and nerve activity quantified relative to baseline firing at time "0" (left). Example recording from a TNBS-treated animal (right) showing the neurogram (top), firing frequency (middle) and pressure channel (bottom). (A2) The response profile of small intestinal nerves was similar between healthy and TNBS-treated animal from two different sources. (A3) The overall excitatory effect was not different between mice from two different suppliers and was not affected by TNBS treatment (top). The inhibitory effect (bottom) was alleviated in TNBS animals from the Jackson laboratory. Overall (A3, right), TNBS-treatment tended to reduce the excitatory and inhibitory effects of bath-applied SSA.

(B1) Mechanosensitivity was alleviated by SSA over time. At 15 min, TNBS-treated animals displayed a higher distension response than healthy animals (middle panel). (B2) Quantification of the overall distension-induced firing at 15 min.

N (SAHMRI, HC) = 6, N (SAHMRI, TNBS) = 10, N (Jax, HC) = 6, N (Jax, TNBS) = 6

Serosal application enables a direct interaction between bacterial mediators and sensory afferent nerves and represents an infection induced by translocation of the bacterium or *S. aureus* bacteraemia. *S. aureus* can also colonise the intestinal lumen. Under these conditions, neuromodulation by *S. aureus* requires diffusion of soluble mediators past the epithelium or is mediated by interaction with epithelial cells.

Intraluminal application

We have previously shown that intestinal afferent sensitivity is relatively unaffected by exposure to intraluminal SSA. Even with undiluted SSA there was only a modest change in afferent sensitivity which we interpreted as reflecting a role for the mucosal epithelium in limiting the exposure of underlying nerves to the bacterial mediators located in the lumen (Uhlig, this thesis). We hypothesised that TNBS would attenuate this barrier function and increase the sensitivity to luminal supernatant. We further hypothesised that deterioration of the mucosal barrier over time in the in-vitro preparation would enhance the sensitivity even further. We therefore compared the effect of SSA in HC and TNBS-animals with luminal SSA applied early or after a delay of 90 min (Fig 3A1).

When administered early in HC, undiluted SSA (100 %) induced a small increase of spontaneous firing (Fig 3A, 3B). The absolute maximum of the response during the 90 min of application was 162.4 % of baseline (median; Fig 3B2, left). The absolute minimum during that time was not significantly different from the maximum (Fig 2B2, right) which is consistent with a maintained response pattern to intraluminal SSA. In TNBS-treated mice, the SSA-induced increase of nerve activity, as determined by the maximum, appeared to be higher than in HC but this was not significant ($median_{TNBS}$= 211.7 %, $p = 0.082$ versus HC). Similarly, the absolute minimum was not significantly different from HC ($p = 0.1255$) and also not from the maximum in TNBS-treated animals again confirming a sustained response to intraluminal SSA. With regard to mechanosensitivity, during early application we observed that the distension-induced increase of nerve activity was well-maintained throughout the duration of the experiment. A small decrease of the area under the curve was observed which was not significantly different between TNBS and HC animals (Fig 3C1.1) which displayed a particularly large variability in the level of mechanosensitivity during SSA application (Fig S3, Fig 3C1.1). This decrease was attributable to a non-significant reduction of the low threshold component (Fig 3C1.2). Together, this suggests that the epithelial barrier in the small intestine is not profoundly affected by TNBS colitis.

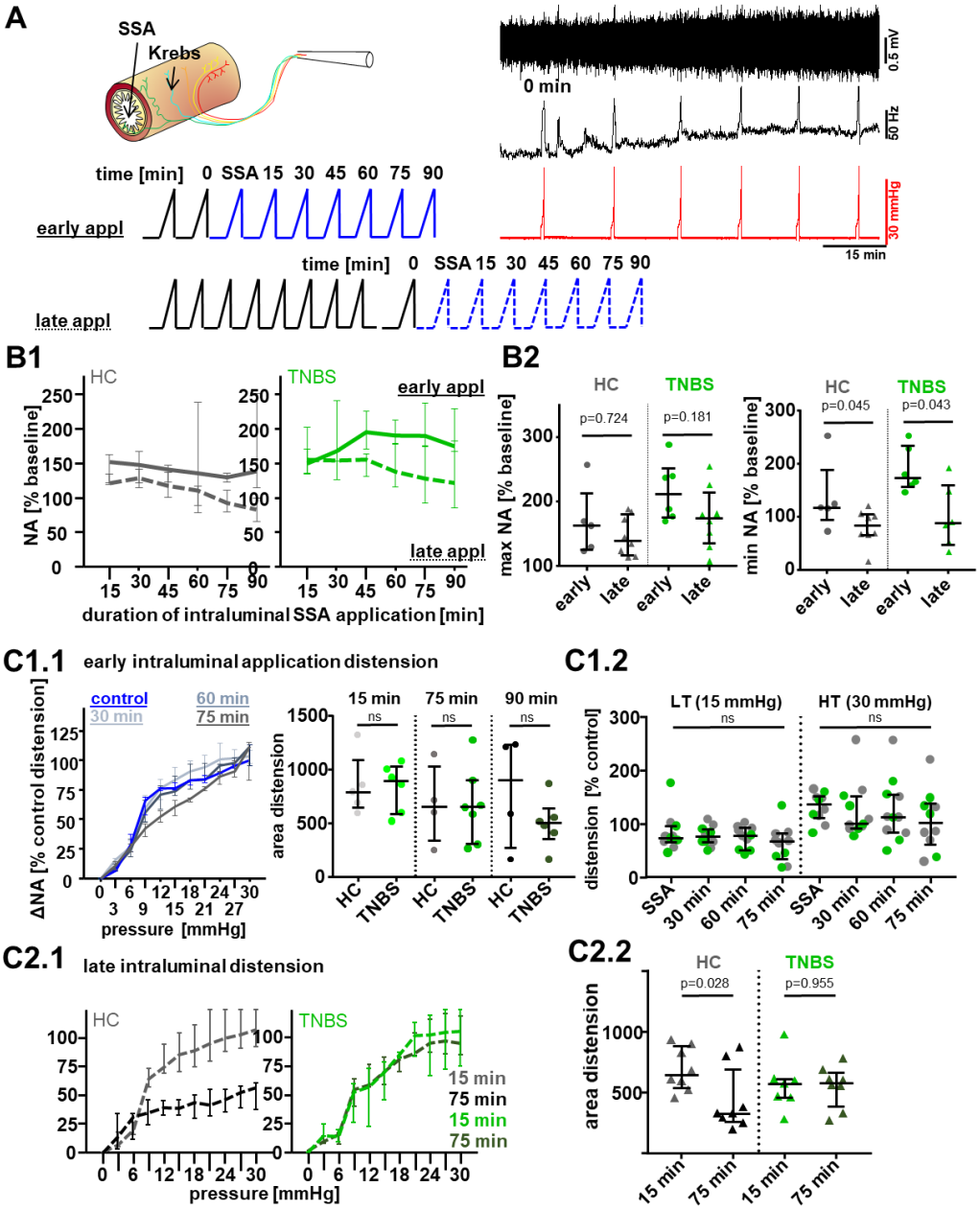

Figure 3: Effect of colonic TNBS treatment on small intestinal nerve response to intraluminal SSA.

(A) Schematic representation of experimental conditions (left). SSA (100 % v/v) were perfused through the intestinal lumen either directly after nerve stabilisation (early application) or after distensions under control conditions (late application). Example trace for early application (right) showing afferent discharge (top), firing frequency (middle) and pressure channel (bottom).

(B1) In healthy and TNBS treated animals, the response profile to intraluminal SSA were affected by prior control distensions.

(B2) The maximal nerve activity was not significantly different between early and late application but tended to be higher in

TNBS-treated animals (left). The minimum nerve activity during intraluminal SSA application was significantly lower when they were applied after control distensions in healthy and TNBS-treated animals.
(C1.1) Mechanosensitivity was not profoundly affected by early intraluminal SSA application. TNBS-treated and healthy animals were pooled (left) because there was no significant difference between them at any time point (right and Fig S3). (C1.2) Both, low and high threshold component tended to be reduced but this was not significant.
(C2.1) Distension-induced increase of nerve activity in healthy animals decreased during late intraluminal application of SSA but not in TNBS-treated animals. (C2.1) Late intraluminal SSA application decreases mechanosensitivity in healthy animals but not in TNBS-treated animals. The overall increase of distension-induced firing in healthy animals was smaller at 75 min than at 15 min.
P values from non-parametric statistical testing (Mann-Whitney or Kruskall-Wallis test with Dunn's Posthoc Analysis), N (HC, early) = 5, N (TNBS, early) = 6, N (HC, early) = 8, N (TNBS, late) = 8

We used repeated distensions to impair epithelial integrity and found that this altered the response to subsequent intraluminal application of undiluted SSA (Fig 3B1). As can be seen in Fig 3B2, the absolute maximum during late intraluminal application tends to be smaller (left) than during early application in both HC and TNBS-treated animals. The minimum was significantly reduced during late application (right) which indicates that the extended protocol alters the permeability of the epithelium for inhibitory mediators. Indeed, we also observed a decrease of mechanosensitivity in healthy control mice (Fig 3C2.1, left). To our surprise, this decrease of the distension response was not observed in TNBS-treated animals after 75 min of intraluminal SSA application (Fig 3C1.2). This could be indicative of an enhanced barrier function as a result of the inflammatory response induced by TNBS treatment.

4 Discussion

These findings provide insight into the changes in afferent sensitivity that accompany inflammation. Firstly, colonic inflammation does not appear to influence sensory signalling from the small intestine. Secondly, following TNBS colitis there are subtle changes in the ability of the small intestinal mucosa to protect against the influence of mediators from pathogenic strains of SA to inhibit afferent firing.

4.1 Is hypersensitivity localised to the site of inflammation?

One hallmark symptom of Irritable bowel syndrome (IBS) is chronic abdominal pain associated with hypersensitivity of colonic sensory afferents to physiological stimuli (Farzaei et al., 2016; Gebhart and Bielefeldt, 2016). In addition, it is beginning to emerge that the small intestine could also importantly contribute to disease pathogenesis with bacterial overgrowth representing a risk factors for the development of IBS (Pistiki et al., 2014).

We used the TNBS model of chronic visceral hypersensitivity to test whether the afferents innervating the small intestine also develop hypersensitivity after acute colitis. In two sets of experiments with C57Bl/6 mice from different suppliers, we found that administration of TNBS 21-28 d before the experiment did not increase the spontaneous discharge or distension-induced firing of small intestinal afferents. This finding was unexpected given the growing recognition of cross-organ sensitization particularly between organs supplied by pelvic afferents, e.g. bladder and colon (Grundy and Brierley, 2018). While TNBS applied to the colon or ileum gives rise to colitis and ileitis respectively and results in hypersensitivity of nerves or neurons supplying this region (Hughes et al., 2009; Moore et al., 2002; Osteen et al., 2016; Stewart et al., 2003), the extent to which one region can influence the innervation of the other has been poorly investigated. For example, inflammation in the ileum (TNBS ileitis, *Trichinella spiralis*) was found to increase the pseudo-affective response to colorectal distension (Bercík et al., 2004; Shah et al., 2016; Tahir et al., 2016). In both of these cases however, gastroenteritis is likely to also have effects on the colon e.g. through leakage of TNBS or anal transfer of antigens. Our approach therefore was to investigate small intestinal hypersensitivity after acute colitis.

We have found that colitis fails to trigger small intestinal hypersensitivity and this suggests that hypersensitivity is localised to the site of inflammation or to regions with overlapping sensory innervation. In this respect, there is a viscerotopic organisation of the afferent innervation to the GI tract (Blackshaw et al., 2007). Proximal regions are supplied by vagal and thoraco-lumbar afferents while distal regions are supplied predominantly by lumbosacral DRG (Kyloh et al., 2011; Peeters et al.,

2006a; Tan et al., 2008). For example, injection of CTB into the jejunum labels neurons between T8-T13, while neurons innervating the colorectum are located in L6-S1. Depending on the injection site (proximal, mid, distal colon), different proportions of TL and LS innervation have been reported (Christianson et al., 2006a). Thus, it is only in the TL region where colon- and small intestine-innervating neurons are located in close proximity which is thought to be required for cross-organ sensitisation. However, the findings that splanchnic high threshold afferents (arising from the TL) particularly contribute to colonic hypersensitivity (Hughes et al., 2009) and that it is partially attributable to modulation of the ion channel TRPA1 which is expressed in small intestinal afferents (Dai et al., 2007; Engel et al., 2011; Peeters et al., 2006a), raises the question why small intestinal afferents would be protected.

The precise location of the neuronal cell bodies innervating a particular intestinal segment cannot be determined and therefore, it is possible that we recorded the activity of fibers which originate from neurons located in far distance to those that innervate the colon. Thus, consistent with the described viscerotopic organisation of DRG and the vagal contribution to small intestinal innervation, those would not be sensitised. Another explanation could be that the cholinergic anti-inflammatory pathway that is exerted by vagal fibers antagonises sensitisation (Jin et al., 2017). Alternatively, it is only the response of a subset of fibers to a specific mechanical stimulus that is affected by intracolonic TNBS. Indeed, Hughes et al. (2009) described that the response of serosal afferents to focal compression increased after TNBS treatment but muscular or mucosal responses to stroking or stretch remained unaffected (Hughes et al., 2009). We employed ramp distensions up to 30 mmHg to assess hypersensitivity and thus, did not distinguish the responses to different mechanical stimuli. This setup is more closely to an in-vivo setting than the flat sheet preparations that have been used previously. Others unsuccessfully attempted to use small intestinal tissue in flat sheet preparations (Rong et al., 2004) and we did not determine whether whole colonic nerve bundles display hypersensitivity under the experimental conditions of this study. Novel imaging techniques that allow to record the responses of individual dorsal root or nodose ganglia neurons during stimulation of the small intestine could be used to investigate the effects of colonic TNBS treatment on the sensitivity of individual small intestine-innervating neurons (Hibberd et al., 2016).

4.2 Effect of TNBS treatment on nerve responses to SSA

Bath-applied bacterial supernatants have a marked effect on afferent sensitivity causing a biphasic response pattern characterised by excitation followed by profound inhibition which in many cases causes complete suppression of spontaneous firing and sensitivity to distension. In sharp contrast, intraluminal application does only induce a small increase of afferent discharge even at the highest concentration which reverses into inhibition only if the epithelial barrier is impaired. Under these conditions, we also observed a decrease of mechanosensitivity during intraluminal application. TNBS treatment did not profoundly change the response patterns to bath-applied or intraluminally perfused SSA. There was small delay of the inhibition of mechanosensitivity in TNBS-treated animals during bath application and late intraluminal application. We were surprised by this finding given the evidence that prior inflammation can alter afferent sensitivity to a variety of mediators acting on ion channels and receptors expressed by sensory neurones (Brierley and Linden, 2014; Campaniello et al., 2017). A repeat study on the same strain of mouse from a different supplier however provided essentially the same outcome but we observed a subtle alleviation of the degree of SSA-mediated inhibition of spontaneous discharge in TNBS-treated mice. We speculate that this may be an adaptation following TNBS colitis which, by altering mucosal permeability, leads to exposure to commensal bacteria, leading to protection against subsequent exposure. Indeed, an increase of intestinal permeability in non-inflamed regions of Ulcerative Colitis patients as well as an increase of the number of goblet cells and a decrease of small intestinal absorption after colonic instillation of TNBS have been described (Amit-Romach et al., 2006; Fries et al., 1999; Gustafsson et al., 2012; Mourad et al., 2017; Torres et al., 1999). Our finding that it was the inhibitory activities of SSA that were alleviated in TNBS animals indicates that these adaptive changes particularly affect the bacterial mediators contributing to the inhibitory effects. Unpublished data from our lab suggests that those constitute short amphipathic peptides that can be inactivated by serum lipoproteins (Surewaard et al., 2012).

It remains to be determined whether a dampened response is beneficial in the context of gastrointestinal infection in IBS. On the one hand, a primed response may be more efficient at eliminating potential threats for example by inducing B cell differentiation and secretion of antibodies. On the other hand, the dampening of the response could also contribute to an incomplete removal of pathogenic bacteria by resident phagocytes leading to persistence of the bacteria in the intestine. Colonisation of nose and intestine with *S. aureus* is a well-known risk factor for *S. aureus* infection (von Eiff et al., 2001; Nouwen et al., 2005).

4.3 Factors contributing to the development of hypersensitivity

It is described that genetic, environmental and experimental factors have a strong impact on the development and maintenance of hypersensitivity in the acute and chronic stage of TNBS colitis (Wirtz et al., 2017). We used a previously established protocol (Castro et al., 2013, 2017; Hughes et al., 2009) to induce hypersensitivity in C57Bl/6J mice from an in-house breeding colony or the Jackson laboratory which were kept under specific pathogen-free conditions in our study but did not find hypersensitivity of afferent nerves in the chronic phase (28 d) in either of these animals. C57Bl/6J mice are traditionally thought to be more resistant to TNBS-induced hypersensitivity because of their preference to develop a T_H1-driven immune response which may, in contrast to a T_H2 response, reduce the susceptibility to chronic visceral hypersensitivity. Additionally, cleanliness could potentially impair the development of visceral hypersensitivity. The reduced levels and composition of environmental bacteria in some facilities may affect the microbiome, the immune response and the degree of inflammation during the acute and chronic stage (Bleich and Fox, 2015; Laukens et al., 2016). Furthermore, hypersensitivity in the small intestine might follow a different time course compared to colonic afferents. In the colon, hypersensitivity is maintained for up to 28 d (Feng et al., 2012; Hughes et al., 2009) but is possible that hypersensitivity of small intestinal nerves resolves earlier because of the higher turnover and the presence of lymphoid Peyer's Patches in the small intestine. The contribution of these factors (genetic background, the microbiome and the time after TNBS treatment) to small intestinal hypersensitivity requires further investigations. In these studies, different aspects of visceral hypersensitivity such as cytokine levels, histological damage and electromyograms in response to colorectal distension should be determined in parallel in addition to small intestinal afferent nerve responses to ensure general hypersensitive phenotype of the animal.

In summary, our findings support the hypothesis that cross-organ sensitisation requires anatomical proximity of the sensory neurons innervating the affected organs in DRG but not proximity of the organs themselves. Furthermore, it appears that systemic and/or gastrointestinal signals contribute to adaptive changes of small intestinal physiology in response to colitis that may impact the response to bacterial mediators.

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6 Supplementary figures

Fig S1

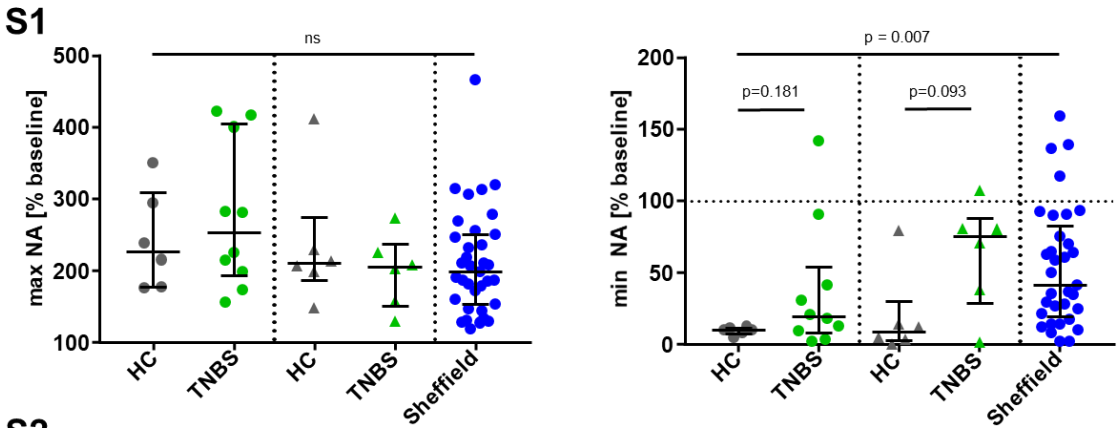

Figure 4(S1): Comparison of the maximal excitatory and inhibitory effect induced by bath application of 20 % SSA in different animal cohorts

Left: The maximal activity induced by SSA was not significantly different between animals from three cohorts (circles represent animals from the SAHMRI in-house colony, triangles animals from the Jackson laboratory). Furthermore, TNBS treatment also did not affect the excitatory effect of SSA.

Right: The animal cohort and TNBS treatment significantly impacted the inhibitory effect of SSA. However, pairwise comparisons between TNBS-treated and healthy SAHMRI (circles) or Jackson (triangles) animals was not significant. The variability of the minimal nerve activity was higher in treated compared to healthy animals and the largest in animals from the Sheffield SPF facility

Fig S2

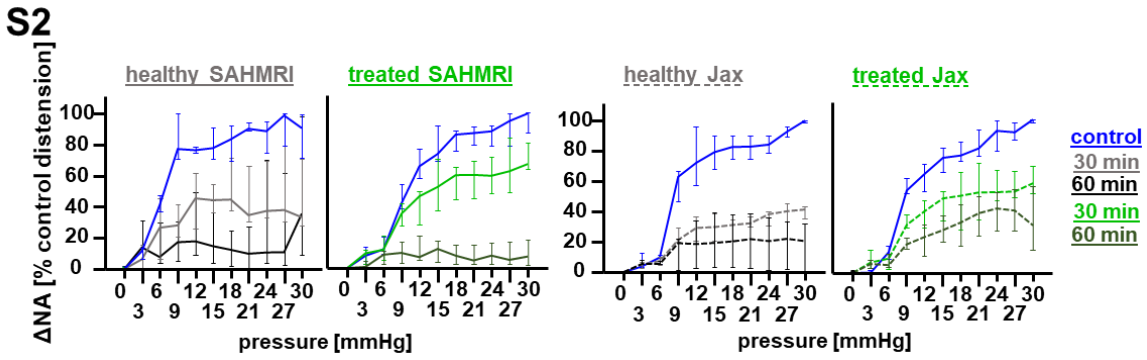

Figure 5(S2): Mechanosensitivity during bath-applied SSA. In animals from both suppliers, distension-induced increase of nerve activity was higher in treated compared to healthy animals at 30 min of application.

Fig S3

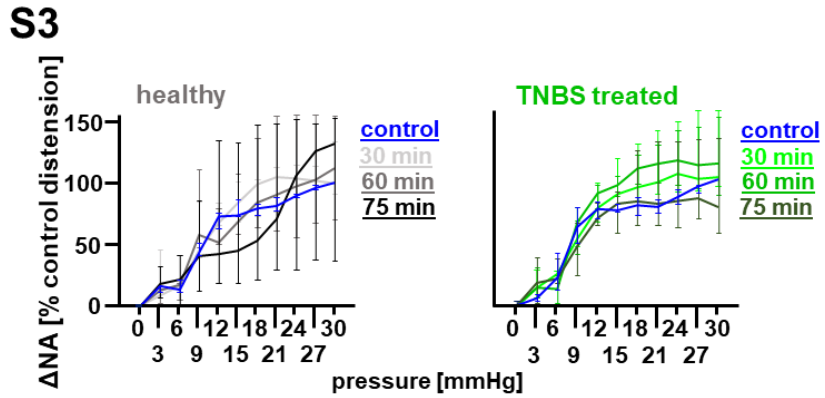

Figure 6(S3): Mechanosensitivity during early intraluminal application. In healthy and TNBS-treated animals, there was no significant effect of SSA on distension-induced firing. However, the mechanosensitivity of healthy but not TNBS-treated animals displayed a large variability.

CHAPTER IV

Identification of soluble mediators produced by *Staphylococcus aureus* that modulate afferent sensitivity and intestinal function

***ABSTRACT*:**

Gastroenteritis is frequently associated with nausea, vomiting, pain and sickness behaviour. Those require activation of central nervous system (CNS) structures involved in reflex circuits and sensation. We have shown that *Staphylococcus aureus* (SA) produces soluble mediators that modulate intestinal afferents and function and now aimed to identify the components contributing to these activities.

Supernatants from various strains of *S. aureus* (SSA) were prepared and bath-applied to small intestinal afferents in an *ex-vivo* preparation of adult male C57Bl/6 mice. Sensory neurons from nodose and dorsal root ganglia were isolated and the effect of SSA on cell permeability investigated using live cell propidium iodide (PI) imaging. Visceral neurons were identified using ip injection of fluorescently labelled cholera toxin. The expression of receptors for soluble mediators was investigated using qRT-PCR. Modulation of intestinal function was assessed in motility experiments and Ussing chambers.

We found that the biphasic response of afferent nerves to SSA consisting of excitation which is followed by inhibition was most pronounced in SSA from strain JE2. It was reduced following heat treatment (90 °C) and dependent on mediators released at high populations density. In a microbiological approach, we found that SSA from JE2 mutants devoid of α-haemolysin (Hla) and phenol-soluble modulins (Psm) lacked the excitatory and inhibitory component of the response respectively. Psms also contributed to the SSA-induced increase of PI fluorescence but were not predominantly involved in SSA's prosecretory effect in Ussing chambers. Receptors for Psms and Hla (formyl peptide receptors and A Disintergrin And Metalloprotease) were expressed in sensory neurons. Other strains of SA (Newman and SH1000) also affected afferent firing and intestinal function but these effects were different from JE2.

These findings indicate that it is virulence factors that contribute to the effects of SSA on afferent nerves and intestinal function which suggests that symptoms associated with infection can be induced by these mediators. This possibility should be addressed in future studies.

1 Introduction

The detection of potentially harmful stimuli is an important function of the peripheral nervous system. Activation of specialised somatosensory fibres of the PNS by stimuli with high intensity induces acute pain and initiates escape reflexes (Woolf, 2018). Similarly, visceral fibres transmit information from internal organs to the brain but the function visceral pain is less clear. It is however a major concern for human health and severely impact patient's quality of life (Farzaei et al., 2016; Labus et al., 2008). Pain from the viscera is often associated with altered bowel habits, nausea as well as vomiting and although these conditions are generally not life-threating conditions, they lead to absence from work, social isolation, etc. (Camilleri and Williams, 2000; Quigley et al., 2006) and therefore, there is a need to better understand the mechanisms underlying the activation of sensory nerves innervating internal organs (Furness et al., 2013; Gebhart and Bielefeldt, 2016).

Acute intestinal pain can be caused by spoiled or contaminated food and gastroenteritis is also a known risk factor the development of chronic visceral pain (Rinttilä et al., 2011). Until recently, it was thought that pain during infections is the result of an over- or inappropriate activation of the immune system. In this model, inflammatory cytokines released by immune cells activate sensory nerves (Campaniello et al., 2017; Hughes et al., 2013). While this mechanism likely contributes to late stages of inflammatory pain, recent studies suggest that pain sensation and other symptoms occurring at early stages of infection are the result of a direct interaction between bacteria or other microbials and neurons (Blake et al., 2018; Chiu et al., 2013).

The possibility of a direct interaction between neurons and bacteria is particularly relevant for the intestine. On the one hand, the intestinal microbiota constitutes a large reservoir of bacteria, viruses, archea and fungi that could potentially interact with neurons (David et al., 2014a; Kelly et al., 2017). On the other hand, the intestine is highly innervated by neurons of the peripheral and enteric nervous system (PNS, ENS). The cell bodies of peripheral nerves are located in nodose and dorsal root ganglia in close contact with the central nervous system. They constitute a population of small to medium-sized neurons with low conductance velocity (Aδ and C fibers) that are mechanosensitive but also express receptors for nutrients and irritants (Brierley and Linden, 2014). The enteric nervous system can also detect these stimuli and is important for the regulation of intestinal motility and secretion (Furness et al., 2013). Interaction of bacteria with neurons from either of these systems could thus contribute to the diverse symptoms during bacterial infections.

Data from several studies support the idea that intestinal sensory neurons from ENS and PNS can be directly affected by mediators from bacteria. For example, data from Kunze and Bienenstock suggest that the activity of PNS and ENS neurons can be modulated by some *Bifidobacterium*, *Lactobacillus* and *Bacteroides* species (Kunze et al. 2009; Wang et al. 2010; Wang et al. 2009). They found that a component of the Bacteroides cell wall, polysaccharide A, induced spontaneous firing and sensitisation of intrinsic sensory neurons (Mao et al., 2013). Others report that lipopolysaccharide (LPS) and other bacterial compounds activate neurons of the PNS, cause the release of pro-inflammatory CGRP and induce nocifensive pain (Chiu et al., 2013; Meseguer et al., 2014). These findings indicate that bacterial cell wall components are important for host-bacteria interaction particularly with regard to bacteria of the intestinal microbiome. However, cell wall glycolipids such as LPS, PSA and proteoglycans are integral parts of the all bacterial cell walls and although they differ between bacteria (Baik et al., 2015), it is unlikely that these are the only mediators that distinguish commensal and pathogenic bacteria. It is enormously important for the host to identify potentially harmful intruders to initiate appropriate defence mechanisms. The production of toxins is a common feature of many pathogenic bacteria but surprisingly, little is known about their interaction with sensory neurons. Recently, Chiu et al. (2013) showed that co-culture of sensory dorsal root ganglia neurons with pathogenic *S. aureus* increases intracellular calcium and found that this was due to the release of α-haemolysin and formyl-peptides (Chiu et al., 2013) which are known to contribute to the pathogenicity of *S. aureus* (Wang et al. 2007; Rauch et al. 2012). This suggests that neuronal detection of bacterial toxins and the pain response may contribute to the host's ability to distinguish pathogenic and commensal bacteria.

The production of many *S. aureus* (SA) virulence factors is regulated by two-component regulatory systems in response to bacterial growth, nutritional cues and other stressors (Burgui et al., 2018). The accessory gene regulator (agr) is the major quorum sensing system in SA (Le and Otto, 2015). During exponential growth, autoinducing peptide (AIP) is secreted and activates the transcription factor agrA via the receptor histidine kinase agrC. The phosphorylated transcription factor upregulates the production of many extracellular virulence determinants via RNAIII. This regulatory RNA modulates the stability of target gene's mRNA and thus, agrA signalling increases the abundance of haemolysins (α-, β- and γ-haemolysin), leucocidins (Panton–Valentine leukocidin (Pvl), LukAB and LukED) and staphylococcal enterotoxins (sea, sea, seb, sec, sed, see) indirectly (Le and Otto, 2015).

The intestine harbours large amounts of commensal bacteria that are beneficial for the host and therefore, the identification of pathogenic bacteria is particularly challenging in this organ. The fast

onset of pain, nausea and vomiting during gastrointestinal infections suggests that extrinsic sensory nerves are able to detect pathogenic bacteria. This prompted us to investigate whether this is indeed the case and applied soluble mediators from *S. aureus* to intestinal afferent nerves. As described previously, supernatants from *S. aureus* cultures (SSA) induced profound changes of small intestinal nerve activity when they were able to make direct contact with these nerves implicating that some components in these supernatants could contribute to the symptoms during *S. aureus* infection. We now aimed to identify the components of SSA contributing to SSA-induced neuromodulation. Using a bacteriological approach, we were able to identify that certain components mediate distinct effects of SSA on nerve activity. The pore-forming toxin α-haemolysin contributed to SSA-induced excitation whereas phenol-soluble modulins were found to inhibit nerve activity. These but also other mediators were also involved in the effects of SSA on intestinal secretion and motility.

2 Methods

Mice

In this study, experiments were performed at the University of Sheffield (UK) and the South Australian Health and Medical Research Institute (SAHMRI, Adelaide, Australia). All animal experiments were performed according to the Animal Scientific Procedure Act 1986 (UK) and the Animal Ethics Committee of The University of Adelaide (Australia). Throughout the study, adult male C57Bl/6 mice (> 10 weeks) were used and kept under specific pathogen-free conditions. They were fed a standard chow and housed in group cages with up to 5 animals.

Retrograde tracing from the viscera

Conjugated cholera toxin subunit B (CTB-488, 250 µg) was injected into the lower left quadrant of anaesthetised C57Bl/6 (2-4 % isoflurane) animals to enable identification of neurons that innervate the intestine for PI live imaging and qRT-PCR (Peeters et al., 2006b). Mice were subcutaneously injected with analgesic (Buprenorphine; 2.7 µg/30 g) and antibiotic (Ampicillin; 50 mg/kg) as they regained consciousness and housed individually. Ip injection was chosen because it labels the majority of intestine-innervating neurons and is less invasive than the more targeted approaches that are commonly used to trace colonic and urinary bladder DRG neurons. To label small intestinal neurons, this approach requires multiple injections which exposes the animal to a more invasive surgery that may cause alterations of gene expression and risks rupturing of the tissue as well as, in worst case, death of the animal. After ip injection, animals were monitored and individually housed for 3-5 d. Then,

animals were euthanised according to Australian animal regulations. Labelled neurons were readily identifiable by a green fluorescence.

Afferent nerve recordings

C57Bl/6 mice were humanly killed according by isoflurane inhalation and cervical dislocation (UK) or CO_2 inhalation and exsanguination (Australia). The intestine was removed from the animal and placed into Krebs solution (mM: NaCl 120, KCl 5.9, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 15.4, glucose 11.5, and $CaCl_2$ 1.2) that was gassed with carbogen (95 % O_2/5 % CO_2). Afferent nerve activity was recorded from nerves innervating the distal small intestine as previously described (Rong et al., 2004) and in the supplementary methods. A segment of the distal small intestine with the attached mesenteric bundles (3-4 cm) was isolated, the ingesta was removed and placed into an organ bath chamber that was continuously perfused with carbogenated Krebs solution. Either end of the segment was connected to a tube which allows continuous intraluminal perfusion. The nerve was dissected and placed into the recording electrode. Nerve activity was recorded and distensions were induced by closing a tab that was connected to the anal end of the intraluminal perfusion system. Intraluminal pressure was measured simultaneously and monitored during distension. When intraluminal pressure reached 30 mmHg, the tab was opened to allow evacuation of the accumulated fluid.

After a 45-60 min stabilisation period and at last two reproducible distensions, vehicle (bacterial growth media) and SSA (20 % v/v) were bath-applied for 15 and 90 min respectively during which, tissue was distended every 15 min. The SSA-induced changes of nerve activity (NA) were quantified as described (p 195) relative to baseline i.e. the spontaneous discharge under control conditions (Krebs perfusion). The effect of vehicle was determined accordingly and subtracted from the effect of SSA (normalised nerve activity, NA). The response to distension was quantified in pressure intervals of 3 mmHg using a script kindly provided by CED Cambridge. The pressure-induced increase of afferent firing during SSA or vehicle application was calculated as a percentage of the maximal distension response induced by distension to 30 mmHg under control conditions (ΔNA [% control distension]).

Motility

To record intestinal motility, the proximal colon (3-4 cm) distal from the caecum was dissected from the whole length intestine after its removal from male C57Bl/6 mice. It was flushed with fresh Krebs solution and tied on to the intraluminal perfusion system of an organ bath chamber similar to the one that was used for electrophysiological nerve recordings. The chamber was constantly perfused with carbogenated Krebs and maintained at 33-34 °C. Krebs was also perfused through the lumen at a rate

of 300 µL/min during the first 20-30 min to clean the colon from any remaining ingesta. Then, the intraluminal perfusion rate was slowed down (100 µL/min) and the outflow tab at the anal end of the perfusion system was closed. Pressure was monitored with the help of a pressure transducer and amplification system that was connected to the anal end using a three-way tab. Once the intraluminal pressure reached 3-5 mmHg, the perfusion was stopped and this small distention induced the generation of migrating motor complexes (MMC).

SSA were bath-applied for 60 min after the initiation of MMC. In the subsequent 30 min wash period, Krebs buffer was perfused through the bath. The number and maximal amplitude of contractions within a 15 min interval at the end of the application or wash-out period was determined and compared to the data from our previous study (SSA-JE2 and vehicle).

Preparation of supernatants

Supernatants from bacteria listed in Table 1 (supplementary methods, p 199) were prepared as described recently. Briefly, sterile bacterial growth media (tryptic soy broth, TSB) was inoculated with single colonies from the respective bacteria grown on TSB agar plates. For some bacteria, antibiotics were added to the plates to select for the desired genotype. Starting cultures were grown overnight (37 °C on a rotary shaker) and diluted to an OD_{600} of 0.150 in a 2 L flask. These flasks were incubated for 24 hours (unless stated otherwise) under the same conditions. Centrifugation was used to remove the bacteria and supernatants were stored in 50 mL falcon tubes at -20 °C until usage. In addition, some experiments were performed using brain-heart infusion (BHI) buffer instead of TSB.

When SSA were used in electrophysiology or motility experiments, they were defrosted in a warm water bath and diluted in Krebs buffer (1:20, 5 % v/v for motility and 1:5, 20 % v/v for electrophysiology). They were bath-applied as indicated by exchanging the Krebs buffer in the reservoir for the diluted SSA.

For Ussing Chamber and propidium iodide experiments, SSA were passed through a sterile filter before usages to prevent contamination of equipment with small numbers of bacteria that may potentially not have been removed through centrifugation. Filtered samples were aliquoted and frozen. Before usage, SSA were defrosted at room temperature and diluted in Krebs (Ussing chamber), Hepes buffer (PI live imaging) or tissue culture medium (PI cytotoxicity) by volume.

Isolation of dorsal root and nodose ganglia

Naïve animals or animals that had been injected with the retrograde tracing dye were humanely killed by CO_2 inhalation and exsanguination (Australian regulations) and placed into ice-cold carbogenated

Krebs solution. The nodose ganglia and dorsal root ganglia from the excised spinal were dissected and placed into ice-cold Hanks balanced salt solution (HBSS) for primary cultures or frozen (-80 °C) for expression analysis. Thoracolumbar (T9-L1) and lumbosacral (L6-S1) regions of the spinal cord were marked with a small incision prior to removal of the spinal cord.

Primary cultures of DRG and NG neurons

Thoracolumbar and lumbosacral DRG as well as NG were enzymatically digested at 37 °C (30 min 4 mg/mL collagenase + 4 mg/mL dispase, 20 min 4 mg/mL collagenase only, GIBCO). Afterwards, ganglia were washed twice with HBSS and triturated using fire-polished glass pipettes with descending diameter. Suspensions were centrifuged and the cell pellet resolved in about 300 µL culturing media (DMEM [Dulbecco's Modified Eagle Medium] supplemented with 10 % FBS, 1 % P/S, glutamine, NGF). 20 µL were plated on poly-D-lysine-(800 µg/mL) and laminin (20 µg/mL) -coated 13 mm glass coverslips. Cultures were flooded two hours after plating with culturing media.

For expression analysis, traced NG, TL or LS DRG neurons were manually selected under a fluorescence microscope using a blunted glass pipette mounted onto a micromanipulator within 24 hours after isolation. Pipette tips were crushed into RNA lysis buffer (10 µL) and pooled (200 µL) to investigate a potential selective expression of FPRs, ADAM10 and Sgms1 in these neurons.

Live Cell Imaging

To investigate the effect of SSA on cell membrane permeability, we performed live cell imaging on primary cultures of DRG and NG neurons and HEK293 cells. HEK293 cells were generously provided by Melissa Adams (Grigori Rychkov laboratory, SAHMRI, Adelaide). Cells were incubated with propidium iodide, a red-fluorescent DNA-binding dye (1 mg/mL diluted 1:1.000 in Hepes buffer for 20 min). The coverslip was mounted on to the recording chamber and cells washed with Hepes (composition in mM: Hepes [2-hydroxyethyl-1-piperazineethanesulfonic acid] 10, NaCl 142, KCl 2, glucose 10, $CaCl_2$ 2, pH adjusted to 7.4 with NaOH) using a hand-made perfusion system. Then, cells were incubated with Hepes buffer (+PI) for 10 min and subsequently, SSA diluted in Hepes (+PI) were perfused for 3 min. The perfusion was stopped and the cells incubated with SSA for another 27 min during which they were continuously imaged. Images were taken every 5 s using the 20x objective of a Nikon TE300 Eclipse microscope equipped with a Sutter DG-4/OF wavelength switcher and connected to Photonic Science ISIS-3 intensified CCD camera with Universal Interface Card Data for data acquisition with MetaFlour software. Regions of interest were selected based on cell shape and PI

staining. Only data from cells that displayed neuronal morphology and no intracellular PI was recorded and further analysed with MicrosoftExcel®.

After subtraction of baseline, two parameters were calculated to compare the effects between different SSA. Firstly, Δfluorescence was used as an indicator for the overall effect of SSA and constitutes the average of SSA-induced fluorescence from all selected cells per coverslip. Secondly, response latencies were determined for responding cells which were identified based on the increase of PI fluorescence. Because the lowest concentration of SSA (0.1 %) or 20 % of vehicle did not increase Δfluorescence further than 15, this value was used to calculate the response latency as the time difference between the start of application and crossing the threshold value.

Cell viability

To quantify cell viability, primary neurons from dorsal root ganglia neurons (supplementary methods) were incubated with SSA diluted in tissue culture medium and propidium iodide (1:1000). In contrast to the live imaging approach, images (fluorescent and bright field) were taken manually every 5 min during the incubation and in subsequent analyses (ImageJ software, NIH), cells were manually categorised into live or dead cells based on increases in PI fluorescence and morphology.

Expression analysis

Segments from the distal intestine in PBS were defrosted and RNA isolated using PureLink® RNA Mini kit. For whole DRG, NG and intestine-innervating neurons NucleoSpin® RNA XS kit (Machenery Nagel™) was used because of the smaller amount of RNA. Intestine-innervating neurons were isolated from primary cultures of TL and LS as well as NG neurons obtained from animals that were subjected to retrograde tracing procedures using a glass pipettes on a micromanipulator.

After RNA isolation, expression of target genes (fpr1-3, adam10, sgms1) and house-keeping genes (β-actin, gapdh) was assessed using quantitative reverse transcription PCR with TaqMan® assays (Mm00545742_m1, Mm00522643_m1, Mm00442803_s1, Mm00484464_s1, Mm01962454_s1, Mm99999915_g1, Mm00607939_s1) and EXPRESS One-Step SuperScript® qRT-PCR Kits. Expression of target genes was quantified against the geometric mean of house-keeping genes (supplementary methods, chapter VI, section 5, p 201).

Ussing Chambers

To investigate the effect of SSA on intestinal secretion, we recorded short circuit currents (SCC) in EasyMount Ussing chambers (Physiologic Instruments, San Diego, USA). Mouse small intestinal

mucosa-submucosa explants were prepared by removing the muscle layer under a dissection microscope. The explants were placed into the inserts (area 0.1 mm^2) for the recording chambers, both chambers filled with carbogenated Krebs solution and tissues were allowed to stabilise for 45 min. All recordings were performed at 35 °C, using $Ag/AgCl_2$ electrodes in KCl and in "REM" mode. SSA were applied to the serosal side and the same amount of Krebs was pipetted into the luminal chamber to account for volume differences.

Data acquisition was performed using ACQUIRE&ANALYZE (Physiologic Instruments) which determines transepithelial potential (voltage, V), electrogenic transport (current, I) and tissue resistance (R) simultaneously. SSA-induced changes of circuit current (SCC) were quantified relative to baseline after the stabilisation period. Area under the curve was calculated by addition of individual values until 1500 s of application. The slope of the SSA-induced increase was determined between 100 and 700 s of application as: ΔSCC/600 s.

Statistical analysis

Raw data was analysed using specialised software and further processed in MicrosoftExcel®. For nerve recording experiments, the overall excitatory activity was calculated by addition of time points during which normalised nerve activity increased (30-45 min) whereas time points with decreased normalised nerve activity (60-90 min) were added for the overall inhibitory activity (area under the curve, AUC, Fig S1). SPSS statistics (version 23, IBM) was used for interpretation and data was extracted and transferred into GraphPad Prism™ for statistical analysis and presentation. Non-parametric statistical analyses were performed and data is depicted as median and interquartile ranges.

3 Results

3.1 *S. aureus* secretes heat-labile mediators that excite and inhibit afferent nerve activity

Bath-applied supernatants from *S. aureus* cultures (SSA) have a profound effect on intestinal afferent firing. As can be seen in Fig 1A (left), 20 % SSA (v/v) from the JE2 strain induce an initial increase in spontaneous afferent firing which reversed to a profound inhibition during continuous application (Fig 1A2, right). In a number of cases, this caused complete silencing of afferent firing 90 min after application. The afferent response to distension consisted of a biphasic increase in firing reflecting the

activation of both low and high threshold mechanoreceptors (Fig 1A3). Following 20% SSA, afferent mechanosensitivity (maximal distension-induced firing) was maintained for 45 min (Fig 1A1, left, until 3rd SSA distension). This was followed by a marked inhibition similar to that seen for spontaneous firing (Fig 1A1, left, 4th to 6th SSA distension). We hypothesised that soluble factors released by *S. aureus* could exert a direct influence on afferent signalling from the GI tract. Subsequent experiments were designed to help identify the nature of bacterial mediators that can excite or inhibit afferent function.

Supernatants from *S. aureus* contain a vast array of mediators that interact with the host. In experiments with SSA that were heated to 90 °C for 15 min prior to bath-application (example trace Fig 1A1), the excitatory and inhibitory effect of SSA on spontaneous intestinal afferent firing (Fig 1A2, middle and right) were significantly attenuated. Heat-treated SSA continued to inhibit mechanosensitivity but to a lesser extent than the unboiled control SSA (Figure 1A3, p 117). This indicates that both heat-stable and heat-labile mediators contribute to the effect of SSA on intestinal afferent firing with the later having a predominant effect on spontaneous afferent firing and the former influencing mechanosensitivity.

The response to JE2 showed a high degree of variability in its ability to excite and inhibit afferent firing (Fig 1A2). Some samples caused profound increases and inhibition and others had little or no effect. It is known that the composition of supernatants from bacterial cultures is dependent on environmental cues (Chapman et al., 2017a). We grew JE2 in two different growth media (BHI and TSB) and applied media or SSA-JE2 for 90 min. In both cases, 20 % (v/v) growth media induced an increase of nerve activity (Fig S2A, $median_{TSB}$ = 146.3 % of baseline vs $median_{BHI}$ = 136.6 %, $p > 0.05$) that was maintained throughout the duration of the experiment. SSA obtained after incubation of either of the growth media with JE2 for 24 hours showed subtle differences in their effect on afferent firing, particularly in relation to the degree to which the SSA caused excitation (Fig S2A, $median_{TSB}$ = 184 % of baseline firing vs $median_{BHI}$ = 176 %, $p > 0.05$). Overall, the degree of excitation induced by SSA was significantly higher than the media-induced increase of nerve activity (Fig S2C). This data is in agreement with our previous study and indicates that *S. aureus* produces neuromodulatory substances in both of the commonly used bacterial growth media.

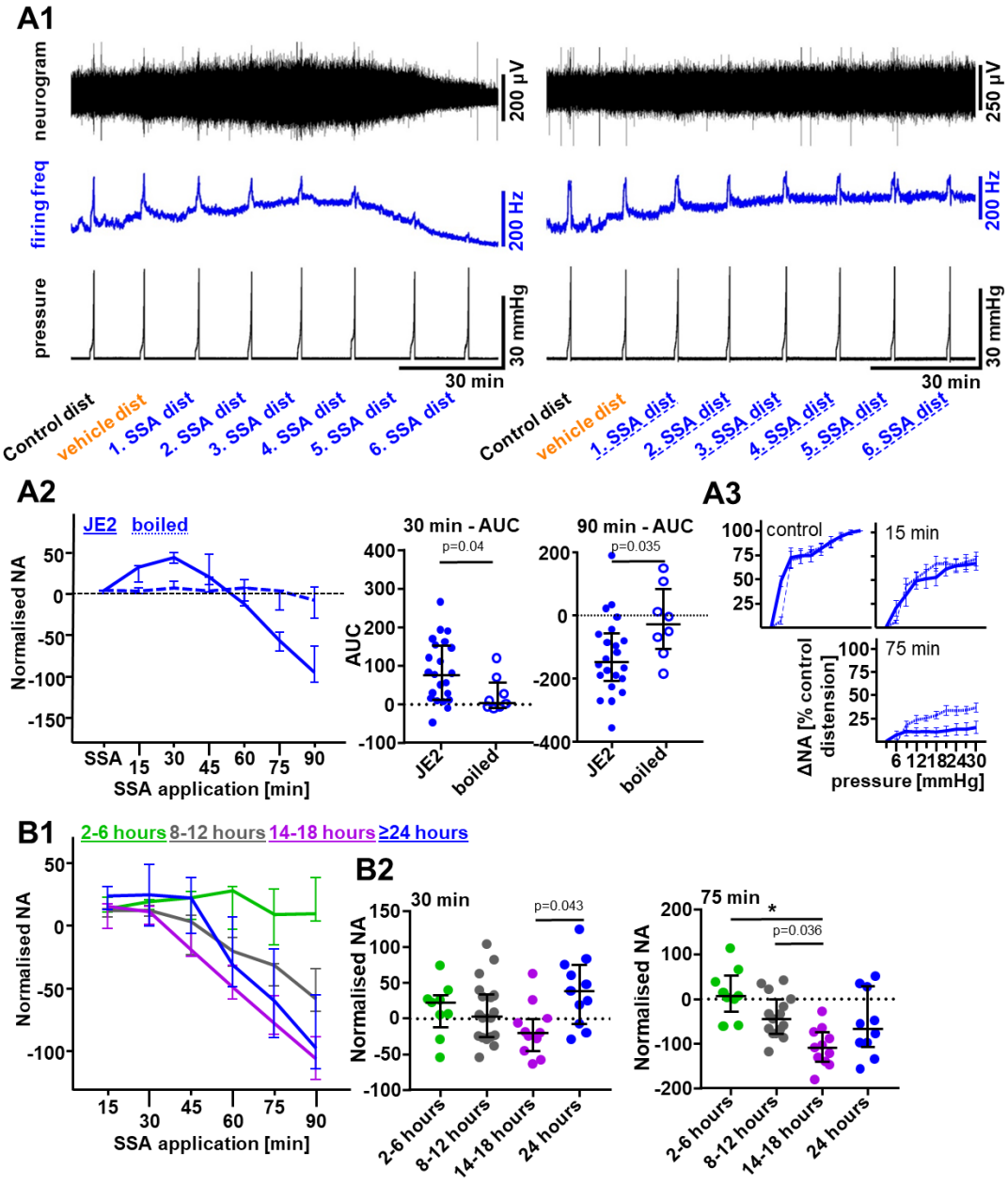

Figure 1: SA produces heat-sensitive soluble mediators at late stages of bacterial growth that modulate small intestinal NA.

(A1) Example traces from nerve recording experiments were SSA (left) or boiled SSA (right) were applied to small intestinal afferent nerves as indicated. Prolonged application of SSA reduced spontaneous firing and mechanosensitivity. Boiling abolishes the effect on spontaneous firing but continues to reduce distension-induced firing. (A2) Response profile (left) of spontaneous nerve activity was changed after heat treatment. Excitatory (middle) and inhibitory (right) effect were significantly reduced. (A3) Mechanosensitivity was partially restored in the absence of heat-labile mediators.

(B1) Response profile of afferent nerves during the bath application of SSA (20 % v/v) obtained after different incubation periods in BHI. (B2) SSA obtained after 24 hours of incubation had the largest effects on afferent NA. SSA from short term cultures (< 6 hours) tended to increase nerve activity at 30 min (left) but did not reduce spontaneous firing at later time points (right).

N(2-6) = 9, N(8-12) = 16, N(14-18) = 12, N(24) = 11, N (≥24) = 14, N(JE2) = 22, N(boiled) = 8

Population density changes the composition of SSA and their effects on afferent nerve activity

Population density is a particularly strong stimulus that induces major changes of the expression profile particularly with the amount of secreted proteins in SSA. In this respect, we investigated the effects of SSA on afferent firing when they were collected at different population density. In culture, population density increases over time and can be determined by measuring the optical density (OD_{600}) of the cultures before centrifugation (Fig S2C). The S-shaped OD_{600}-time relationship represents log, exponential and stationary phase of bacterial growth and therefore, time and OD_{600} can be used as surrogates for the growth stage. In experiments with SSA obtained after different incubation time, SSAs from 24 hour cultures induced the largest excitation at 30 min (Fig 1B2, left). Interestingly, SSA obtained after shorter incubation time (< 6 hours) also tended to increase nerve at this time point. In contrast, only SSAs from long-term cultures (> 14 hours) significantly inhibited spontaneous firing during continuous application (Fig 1B2, right). The inhibitory effect was also linked to optical density. Only SSA with high optical density (> 10) significantly decreased nerve activity (Fig S2B). This suggests that it is *S. aureus* mediators released during the stationary growth phase that exert a profound dual influence on intestinal afferent firing. At high density (stationary phase), quorum sensing (QS) increases the expression of secreted protein whilst simultaneously decreasing the abundance of surface-bound proteins. Our data are consistent with secreted proteins influencing afferent excitability.

The transition from exponential to stationary growth phase and the associated changes of protein expression are regulated by transcription factors that are activated as a consequence of QS. In *S. aureus*, the accessory gene regulator (agr) system is particularly important in this process and regulates the expression of many genes that contribute to the pathogenesis of *S. aureus*. The Centre for Staphylococcal Research created 1952 mutants by transposon insertion (Nebraska library) from the community-associated MRSA strain USA300 (JE2, Fey et al., 2013). Each of these mutants carries a single dysfunctional gene and allows to study of its contribution to disease or in our case afferent sensitivity.

Quorum sensing knock out reduces excitation and inhibition

We prepared SSAs from the AgrA mutant of the Nebraska library (SAUSA_1992) and compared their activity with the wildtype JE2. Remarkably, SSA-$AgrA^-$ (20 % v/v) had no effect on spontaneous afferent firing nor did it affect either the low threshold or high-threshold component of the mechano-sensitive response to distension (Fig 2A1). This complete dependence on agrA is consistent with the finding that stationary phase mediators are necessary for any modulation of afferent firing and indicates that QS-regulated components are critical for neuromodulatory mediator release.

3.2 Investigation of individual components of *S. aureus* supernatants on afferent activity

The remarkable AgrA-dependence suggested that genes contributing to the pathogenesis of SA are important for the SSA-induced changes of spontaneous nerve activity and mechanosensitivity. Many agrA-regulated genes constitute virulence factors that are produced in large quantities during *S. aureus* infection. Among them, pore forming toxins (PFT) and bacterial enzymes play a major role during host-invasion of the bacteria and escaping from the immune system (LaSarre et al., 2013). We hypothesised that it is these virulence factors that also contributed to neuromodulation and used single mutants from the Nebraska library to elucidate which mediators were involved in the SSA-induced changes of small intestinal nerve activity. In a candidate approach, we initially focussed on heat-labile components because of our findings using boiled SSA.

α-haemolysin (Hla) contributes to initial increase of nerve activity

The wild type strain of the Nebraska library (JE2) is known for its ability to produce the pore-forming toxin α-haemolysin (Hla). An Hla mutation reduces the severity of infections and recently, Hla has been shown to increase intracellular calcium in sensory neurons and induce spontaneous pain in a *S. aureus* skin infection model (Chiu et al., 2013). This accentuated Hla as a prime candidate for direct effects of SSA on intestinal afferent nerves.

To investigate the role of Hla, we prepared SSA from the Hla-deficient JE2 mutant of the Nebraska library (SAUSA300_1058, SSA-Hla$^-$) and applied them to afferent nerves. SSA-Hla$^-$ application did not induce the initial increase of nerve activity that we observed during SSA-JE2 application (Fig 2A1, left). The integrated excitatory effect of SSA-Hla$^-$ was significantly smaller compared to wildtype SSA (Fig 2A1, middle). In contrast, SSA from wildtype JE2 and the Hla-deficient JE2 both inhibited nerve activity at later time points to a similar extent (Fig 2A1, right) suggesting that α-haemolysin does not contribute to the inhibition of spontaneous firing. Supernatants lacking Hla (SSA-Hla$^-$) also continued to reduce mechanosensitivity (Fig 2A2). This reduction tended to be less pronounced than the inhibition induced by SSA from wildtype JE2 but the difference was not significant at any time or pressure point. Overall, this indicates that Hla does indeed have direct effects on small intestinal nerve activity and is involved in the excitatory effect of SSA but does not to the inhibition of spontaneous firing and mechanosensitivity.

In addition to Hla, JE2 produces other heat-labile pore-forming toxins that have an important role in disease progression. Panton-Valentine Leucocidin (Pvl) has been shown to contribute to *S. aureus* pneumonia in mice (Labandeira-Rey et al., 2007) but its importance in other animal models of infection is still a matter of debate. In contrast, Pvl is a major contributor to human disease. Pvl-producing strains are highly pathogenic and Pvl activates human neutrophils via receptor-dependent calcium mobilisation (Cardot-Martin et al., 2015; Tawk et al., 2015; Werner et al., 2002). Similar pathways could be involved in the modulation of nerve activity and this was tested using the Pvl-deficient JE2 mutant from the Nebraska library.

Supernatants from this mutant (SAUSA300_1382, SSA-Pvl$^-$) induced an initial increase of afferent nerve activity (Fig 2A1, left and middle) which appeared to be smaller than the overall excitatory effect induced by SSA-JE2 (Fig 2A2, middle). The paired comparison of SSA-JE2 and SSA-Pvl$^-$ however was not significant (median 115.5 vs 28.3, $p = 0.059$, Mann-Whitney test). There was also no significant difference between the inhibition of nerve activity (Fig 2A1, right) induced by SSA from Pvl-deficient JE2 and wildtype bacteria (median -97.4 vs -149.3, $p = 0.662$, Mann-Whitney test) suggesting that Pvl does not contribute to SSA-induced changes of spontaneous nerve firing. With regard to mechanosensitivity, we found that SSA-Pvl$^-$ reduced the distension-induced increase of nerve firing to a similar degree as SSA-JE2 at any time point. This is shown in Fig 2A2 by the almost complete overlay of the distension response curves during application of SSA from these two bacteria. Our findings indicate that Pvl is not important for SSA's neuromodulatory effects in our ex-vivo preparation which may reflect the lower sensitivity of murine compared to human primary cells (Spaan et al., 2017).

In summary, we found that SSA lacking individual agrA-regulated components (Hla, Pvl) with known function in *S. aureus* pathogenesis continued to modulate intestinal nerve activity. Only the initial increase of spontaneous firing induced by SSA was reduced in the absence of individual mediators. This suggests that a differential interaction of intestinal nerves with several mediators produced by *S. aureus* is involved in complex effects of SSA on spontaneous nerve activity as well as the reduction of mechanosensitivity.

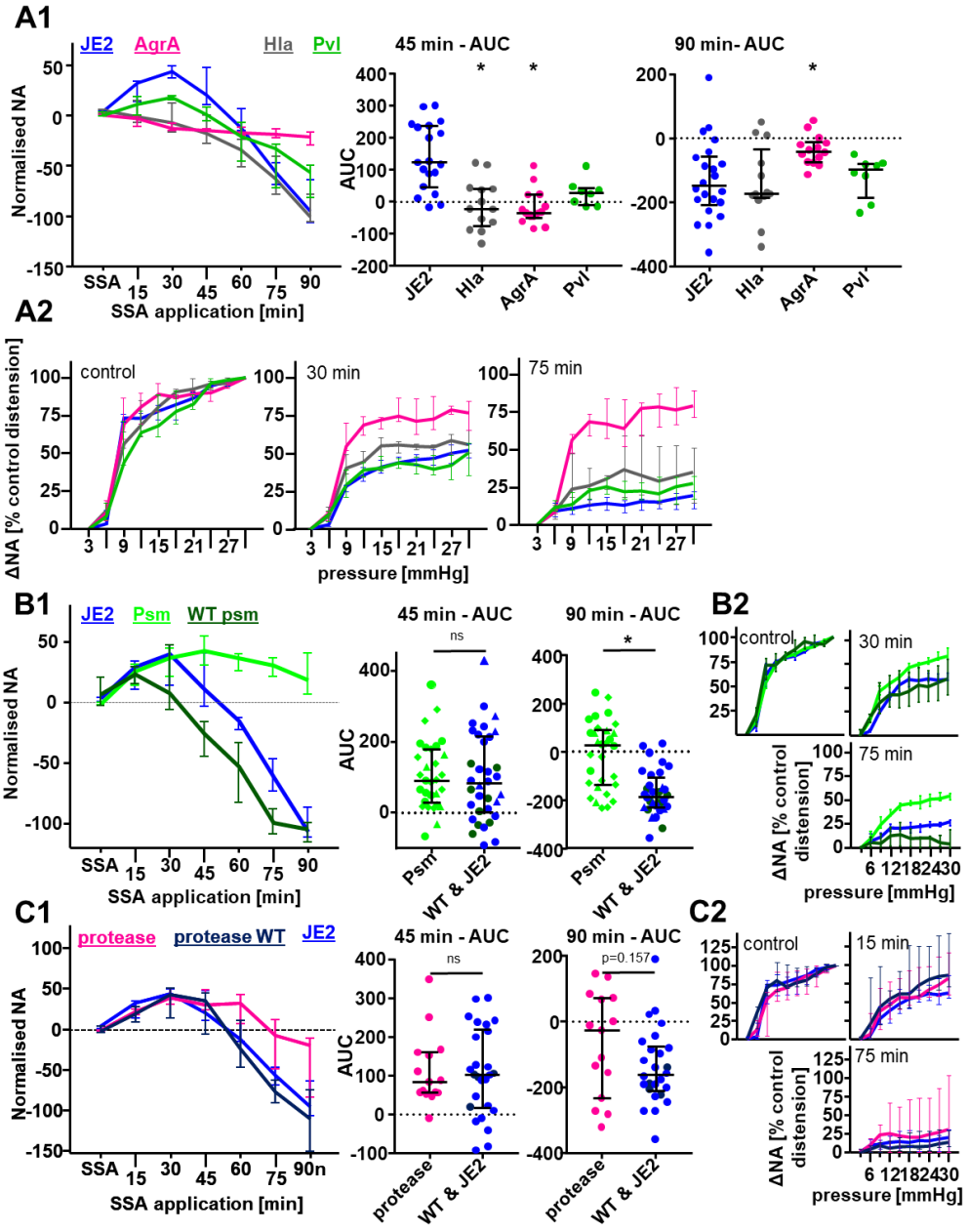

Figure 2: Investigation of the contribution of individual soluble mediators to the excitatory and inhibitory effect of SSA.
(A1) In the absence of the quorum sensing regulator AgrA, excitation and inhibition were significantly alleviated. The absence of individual toxins (Hla, Pvl) decreases the excitatory component which was only significant for Hla. (A3) Mechanosensitivity decreases during incubation with SSA-JE2. This inhibitory effect was alleviated when no quorum sensing-regulated molecules. (B1) Psms mediate the inhibitory effect of SSA JE2 on small intestinal afferent nerve activity. The excitatory effect was not changed in the absence of Psm. (B2) Mechanosensitivity was partially rescued when Psm were not present in SSA-JE2.
(C1) Response profile of NA during application of SSA missing bacterial proteases (left panel). Excitation was not alleviated. Inhibition was not observed in some experiments with protease-deficient SSA but the alleviation was not significant. (C2) Protease-deficient SSA decreased mechanosensitivity similar to wild-type SSA.
N(JE2) = 22, N(AgrA) = 14, N(Hla) = 13, N(Pvl) = 8, N(Psm) = 34, N(Psm WT) = 8; data for figure (B) was obtained in two locations and N(JE2) = 41, N(protease) = 15, N(protease WT) = 4

With our single mutant approach using the Nebraska library, we identified Hla as mediator within SSA-JE2 that acutely increases nerve activity but we were not able to deduce which substances mediated nerve inhibition. We were particularly interested in identifying inhibitory substances because these might constitute an interesting target for drug development. Inhibition of nerve activity can be the result of profound alterations of the membrane potential which can be caused either by blockage of ion channels and transporters or changes of membrane permeability. We hypothesised the latter might be the case because *S. aureus* and the JE2 strain in particular are known to produce amphipathic substances with a high affinity for lipids. Phenol soluble modulins are present in large quantities in JE2 supernatants (up to 62 % of all proteins) and constitute a group of seven short peptides with α-helical structure (Wang et al., 2007). The four α-Psms (20-25 aa), two β-Psms (43-45 aa) and δ-toxin (26 aa) can insert into liquid-disordered membrane domains and cause membrane disruption at high concentrations. The Nebraska library does not contain mutants missing the seven Psm peptides as the encoding genes are short. Additionally, the sequence encoding δ-toxin is integrated into the RNAIII and therefore, untargeted disruption of the δ-toxin would also inactivate the transcription of RNAIII.

Phenol soluble modulins contribute to inhibition of spontaneous firing

To investigate the contribution of Psms to SSA-induced neuromodulation, we used a mutant carrying mutations in the genes for all seven peptides (Wang et al., 2007). It has been generated, validated and generously provided by Micheal Otto (NIH).

Supernatants from this mutant (SSA-Psm$^-$) excited intestinal sensory nerves to a degree which was similar to the wildtype (Fig 2B1, middle). With regard to the inhibition, we observed a particularly large variability of the responses (Fig 2B1, right). In our initial set of experiments (N = 11, data represented as circles), SSA-Psm$^-$ did not inhibit spontaneous discharge during continuous application (9/11, 81.2 %) and we concluded that Psms were responsible for SSA-induced inhibition of nerve activity. In a different cohort of animals, we wanted to identify the subgroup of Psms which was most important for nerve inhibition and prepared supernatants from JE2 mutants lacking either α-Psms, β-Psms or δ-toxin. All of these SSAs (SSA-Psmα$^-$, SSA-Psmβ$^-$, SSA-Psmhld$^-$) induced an inhibition similar to the JE2 wildtype (not shown) and therefore, we repeated the experiments with SSA from the complete knock-out strain (SSA-Psm$^-$) in this cohort of animals (N = 17). In contrast to our initial data, SSA-Psm$^-$ inhibited nerve activity in the majority of these experiments (Fig 2B1, right, data shown as squares). Only 7 out of 17 nerves (41.2 %) were not inhibited. To validate our findings, we performed a final set of experiments on a third set of animals (see methods for differences between animals). Here, nerve activity was not reduced by SSA-Psm$^-$ in 4/6 recordings (66.7 %, Fig 2B1, right, triangles). In

sum, the inhibitory effect of SSA was significantly reduced by the lack of Psms (Fig 2B1, right and Fig S3) and SSA-Psm$^-$ did not inhibit nerve activity in 58.82 % (20/34). This suggests that Psms contribute to inhibition of spontaneous nerve firing, at least in some animals. In others, host-inherent factors the inhibitory effect of SSA was dependent on other mediators.

Host factors appeared to particularly contribute to the susceptibility to Psm-deficient supernatants. The ability of adapt to environmental stimuli is crucial for survival and induces changes of metabolism and gene expression. The intestinal microbiome constitutes a particularly strong factor that influences the expression of many genes in the gut and could be responsible for inter-individual variation. To investigate the effect of bacterial abundance, nerve recording experiments were performed using segments of the proximal colon where bacterial density is higher than in the small intestine. We focussed on SSA-Psm$^-$ because of the pronounced variability of the responses. In experiments with supernatants lacking Psms, we observed an increase of nerve activity (Fig S4). This increase was of a similar magnitude compared to the excitation induced by SSA-JE2. Spontaneous firing increased until about 45 min of SSA-Psm$^-$ application and did not decrease below the discharge frequency at the start of the experiment in the majority of experiments (8/10, 80 %). In contrast, SSA from the JE2 wild type induced a profound inhibition by 60-75 min of application (Fig S4,). Although this data was obtained from different mice, it is unlikely that this is solely responsible for the significant difference between SSA-Psm$^-$ and SSA-JE2. In the small intestine, the nerve response profile to SSA-JE2 was not significantly different between animals from the different cohorts (Fig S1, S3). Our data from the colonic experiments confirm our findings from the small intestine and indicate Psms are important contributors to SSA-induced inhibition of NA.

The higher consistency of the results with Psm-deficient SSA in the colon could also suggest that bacterial mediators preferentially target subpopulations of sensory nerve fibres. In the proximal colon, most sensory innervation derives from cell bodies in the spinal cord while vagal and spinal fibers innervate the small intestine. It is known that these fibers also convey different aspects of intestinal sensing to the brain. Spinal and particularly splanchnic fibers originating from dorsal root ganglia of the thoracolumbar region of the spinal cord are predominantly mechanosensors with high activation thresholds. On the contrary, lumbosacral spinal and vagal afferents have a higher proportion of mucosal terminals and respond to stimuli with lower intensity. Intraluminal distension sequentially activates low threshold, wide dynamic range and high threshold fibers and can be used to distinguish these subpopulations of afferent fibers.

When small intestinal tissue was distended, the pressure-induced increase of afferent discharge was significantly higher during application of SSA-Psm$^-$ compared to wildtype SSA (Fig 2B2), particularly at late time points (75 min, Fig 2B2). This means that the SSA-JE2-induced inhibition of mechanosensitivity was reduced when SSA from Psm-deficient *S. aureus* were applied and indicates that Psms contribute to the inhibition of mechanosensitivity by wildtype *S. aureus* JE2. To investigate whether Psms in SSA-JE2 targeted a particular subpopulation of mechanosensitive nerves, the distension-induced firing was integrated between 0 and 9 mmHg, 12 and 21 mmHg and > 24 mmHg for low threshold (LT), wide dynamic range (WDR) and high threshold (HT) firing respectively (Fig S5A). During early application (2nd distension in the presence of SSA), the distension-induced firing at low pressures was significantly higher when SSA lacking Psms were applied compared to SSA-JE2 (Fig S5B) suggesting that Psms in wild type SSA may particularly decrease the activity of LT fibers during early application. At later time points, the mechanosensitivity of all three components was significantly higher during SSA-Psm$^-$ compared to SSA-JE2 application. In fact, the difference between SSA-Psm$^-$ and SSA-JE2$^-$ was more pronounced for medium- and high pressure-induced firing ($p < 0.001$for WDR and HT, Mann-Whitney test). This indicates that Psms reduce the distention-induced firing of all three populations of mechanosensors. However, as can be seen in Fig S5C, application of SSA-Psm$^-$ continued to reduce mechanosensitivity of WDR and HT components to a similar degree as SSA-JE2 in a small number of experiments. These data were obtained from experiments where SSA-Psm$^-$ induced inhibition of spontaneous nerve activity (squares) but also from experiments without inhibition (circles). This suggest that the effects on spontaneous and distension-induced firing are not necessarily linked to one another.

Our experiments with supernatants lacking Psms indicate that they do not fully account for the reduction of spontaneous nerve activity and mechanosensitivity. Other mediators are likely to contribute to the profound inhibitory effects of SSA.

Neuromodulation by SSA-JE2 was predominantly attributable to the pore-forming toxin Hla (excitation) and Psms (inhibition) but we only observed a complete reversal of all SSA-induced changes of nerve activity the complete absence of agrA. In experiments with the agrA mutant, the responses of individual nerves also tended to be more consistent. This suggested that agrA-regulated mediators other than Hla and Psms contributed to the variability of the responses to SSA and alteration of nerve activity. The proteome of *S. aureus* contains several proteases that are essential for tissue invasion during pathogenesis and those seemed good candidates for three reasons. Firstly, the activity of

proteases depends on the local environment and could therefore contribute to the variability between different segments. Secondly, it is possible that bacterial proteases cleave cell-matrix proteins which are thought to be important for mechanosensitivity (Delmas et al., 2011; Wang et al., 2017). And thirdly, host proteases can induce depolarisation of neurons via protease-activated receptors (PAR) which are also expressed in terminals of intestinal nerves (Adams et al., 2011). Protease-induced cleavage of the extracellular domain generates the receptor ligand and its binding induces conformational changes which lead to the activation intracellular G proteins and depolarisation. Because PARs are quickly internalised upon activation, we hypothesised that activation of PAR and subsequent internalisation could contribute to the increase and decrease of NA respectively.

Bacterial proteases do not importantly contribute to the effects of SSA on nerve activity

An interaction between host PARs and *S. aureus* proteases has not been investigated before and therefore, we obtained a SA mutant missing 10 of the major proteases instead of using individual mutants (Kolar et al., 2013) to investigate whether any of those was likely to contribute to SSA's neuromodulatory effects.

Supernatants from cultures of the protease-deficient mutant (SSA-Prot$^-$) increased nerve activity to a similar extent as SSA-JE2 between 0 and 45 min (Fig 2C1, middle). The onset of inhibition tended to be delayed and alleviated compared to wild type SSA (Fig 2C1, right) but the high degree of variability of the inhibitory response during prolonged SSA-prot$^-$ application does not allow to draw a final conclusion about the contribution to SSA-induced inhibition of spontaneous afferent firing. The profound abrogation of mechanosensitivity induced by SSA-JE2 was also observed when SSA lacking proteolytic activity were applied (Fig 2C2). In contrast to the distension response curves of other mutants however where the reduction of mechanosensitivity tended very consistent, the effect of SSA-Prot$^-$ on distension-induced firing was highly variable. Overall, this suggests that bacterial proteases do not play a major role in increasing or decreasing small intestinal afferent nerve activity themselves and argues against an involvement of neuronal PARs. They may however indirectly contribute to neuromodulation by enabling the tissue penetration of soluble mediators. In the absence of bacterial proteases, nerve responses were highly variable which may reflect tissue integrity that can be affected by physiological factors such as inflammation, stress, diet and the activity of host proteases.

In conclusion, we find that the QS-regulated pore-forming toxins Hla and Psms can alter small intestinal afferent activity and contribute to the different aspects of neuromodulation. The large variability of responses may reflect host factors but also the activity of bacterial proteases.

The USA300 strain of *S. aureus* which we used throughout this study, was isolated from skin and soft tissue infections in 2000 and has since emerged as a major threat to human health. It is known to secrete particularly large amounts of numerous virulence factors and this seems to be essential for its ability to infect otherwise healthy individuals and cause severe diseases in a variety of tissues (Kretschmer et al., 2010; Nishiyama et al., 2012). Genome sequencing and comparison of USA300 with other *S. aureus* strains has been used to identify virulence factors with particular relevance for disease. This data show that virulence factor production is a common feature of pathogenic strains but also some commensal strains of *S. aureus*. Each strain however produces distinct arrays of virulence factors and these genetic differences have probably evolved as a consequence of adaptation of *S. aureus* strains to a specific niche and type of infection (Planet, 2017; Young et al., 2017). For example, hospital-associated *S. aureus* strains produce less toxins and display a reduced capacity to adapt to different environments compared to the community-associated *S. aureus* strains such as USA300 (Otto, 2013). These strains also differ in their ability to induce cytokine release from B and T cells, kill phagocytes and persist in epithelial cells all of which contribute to disease pathogenesis (Strobel et al., 2016). Our findings add modulation of neuronal activity to this list and prompted us to investigate whether, in line with strain-specific effects on other cell types; SSA-induced neuromodulation also differed between *S. aureus* strains.

3.3 The effects of *S. aureus* on small intestinal sensory nerves are strain-specific

The absolute abundance of individual mediators is strain-specific and linked to disease severity. To test whether the response profile of afferent nerves to SSA is related to the pathogenic potential of the parental bacteria, we prepared supernatants from two other strains that are frequently used to study *S. aureus* infections in animal models.

The Newman strain has been isolated from a patient with *Mycobacterium tuberculosis*-induced osteomyelitis in 1952 and carries a mutation in the transcription regulator saeS encoding gene (Baba et al., 2008; Geiger et al., 2008; Lorenz and Duthie, 1952). The two-component system SaeRS regulates virulence factor expression in addition to agrA and the mutation in Newman causes constitutive activation of SaeS and thus expression of its target genes. We therefore expected that application of SSA-Newman would cause a similar or exaggerated response pattern compared to SSA-JE2.

With regard to spontaneous afferent discharge, SSA-Newman induced an initial increase of nerve activity with similar magnitude as SSA-JE2 (Fig 3A1, middle). In contrast to JE2 however, the increase of SSA-induced afferent discharge was maintained until 90 min in 6/15 experiments. In others, afferent discharge reversed to vehicle levels but did not decrease any further. Overall, there was a slight reduction of spontaneous firing at later time points compared to early time points (Fig 3A1, median $AUC_{Newman\ early}$ = 104 vs $AUC_{Newman\ late}$ = 44) but this was negligible and significantly smaller than JE2-induced inhibition (median $AUC_{JE2\ late}$ = -149.3). We hypothesise that this reduction could therefore be due to desensitisation. Alternatively, SSA-Newman might contain mediators that prolong the excitatory effect of SSA. SSA from Newman strain also had a smaller effect on mechanosensitivity than JE2. As shown in Fig 3A2, afferent nerves continued to respond to intraluminal distension when SSA-Newman were applied which is in sharp contrast to the effects of SSA-JE2 that almost completely abrogated distension-induced firing at 75 min (Fig 3A2, 75 min). This suggests that SSA-Newman produces smaller amounts of bacterial mediators that reduce mechanosensitivity. This finding was unexpected because Newman is known to produce comparable amounts of pore-forming toxins (Chapman et al., 2017a) except for Pvl and also secretes Psms (Fig S6) which we found to be involved in JE2-induced inhibition of mechanosensitivity which is consistent with other mediators contributing to inhibition of mechanosensitivity.

The Newman and USA300 strain are both highly pathogenic in animal models but only USA300 inhibited spontaneous and distension-induced firing arguing against inhibition as an indicator of disease severity. In contrast, both strains induced a high degree of excitation. To further investigate whether excitation was linked to strain pathogenicity, we tested a laboratory *S. aureus* strain that is used in models with a less severe progression of disease. The SH1000 strain of *S. aureus* was derived from the NCTC8325-4 isolate in 2002 by mutating a transcriptional regulator (RsbU) of stress factor σ^B (Horsburgh et al., 2002). Compared to NCTC8325-4, the intact stress response in SH1000 reduces the amount of exoproteins. This suggested that SSA from SH1000 would also have smaller neuromodulatory effects than other SSAs.

When applied to intestinal afferent nerves, SSA-SH1000 increased spontaneous afferent firing (Fig 3A1, left). The overall excitatory effect (Fig 3A1, middle) tended to be smaller than the increased induced by SSA-JE2 or -Newman but this difference was not significant because SSA-SH1000 induced a comparable degree of excitation in 4/13 experiments. Similarly, the overall inhibitory response appeared to be smaller in experiments with SSA-SH1000 compared to the JE2 strain (Fig 3A2, right)

but this was also not significant when all three strains were included in the statistical analysis (p = 0.060, Kruskall-Wallis test). The paired comparison between SSA from SH1000 and JE2 with regard to AUC (Fig 3A2, right) and SSA-induced change the normalised nerve activity at 90 min (Fig 3A1) however were both statistically significant (p = 0.030, Mann-Whitney test for AUC and p = 0.020, Mann-Whitney test for normalised nerve activity at 90 min). This indicates that SSA-SH1000 have indeed a smaller neuromodulatory effect than JE2. The distension response curves during SSA-SH1000 application were similar to those during SSA-Newman application (Fig 3A2). Only at later time points (75 min), the distension response curves started to deviate from one another indicating that it is only during prolonged application where SSA from strain SH1000 inhibit mechanosensitivity. The extent of inhibition was smaller and its onset later than the profound inhibition induced by SSA from JE2. These data are consistent with the reported reduced agrA activity and Hla production in this *S. aureus* strain (Horsburgh et al., 2002) and also confirm our earlier results that these substances play major roles in SSA-induced neuromodulation.

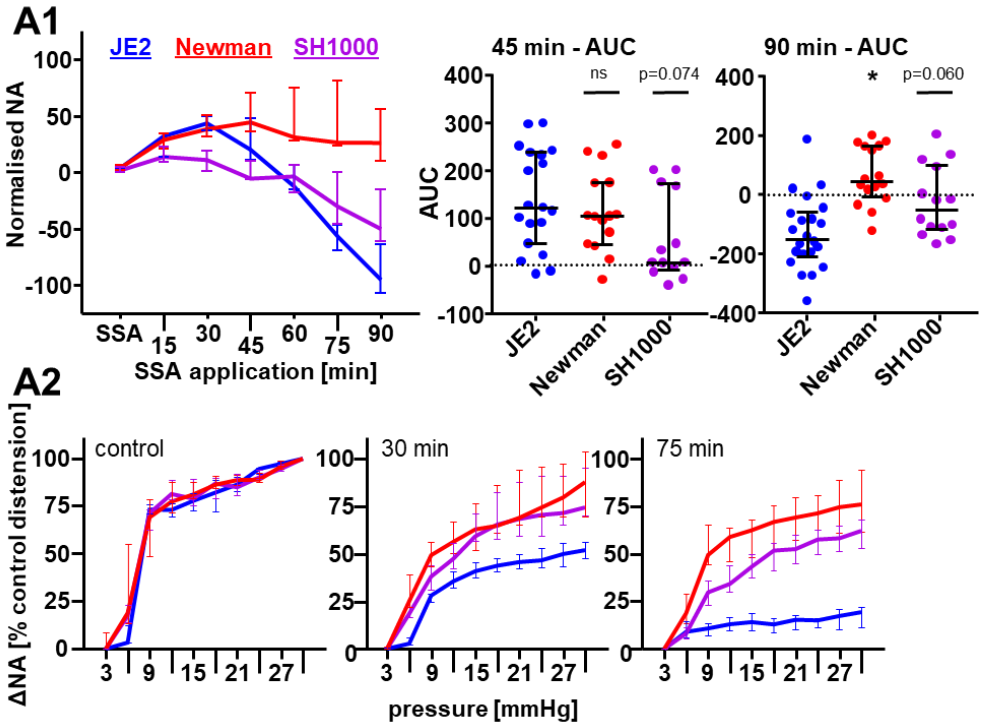

Figure 3: Strains of S. aureus that secrete different arrays of soluble mediators re-produce some effects of SSA-JE2. (A1) The response profiles from the three strains during prolonged application of SSA was different (left). SSA from SH1000 strain tended to produce a reduced level of both excitation (middle) and inhibition (right). In contrast, SSA-Newman excited NA to a similar extent as JE2 but did not result in the pronounced inhibition. (A2) SSA from SH1000 and Newman did not decrease mechanosensitivity to the same extent as SSA-JE2. N(JE2) = 22, N(Newman) = 15, N(SH1000) = 13

Together these findings from different strains are consistent with the phenotype of the parental strain reflecting the neuromodulatory effects of bacterial supernatants. Yet unidentified components secreted by *S. aureus* strain Newman may contribute to a prolonged excitation.

S. aureus strains that cause disease (JE2, Newman and SH1000) were found to have profound effects on intestinal nerve activity. Other strains of *S. aureus* are part of the human and murine microbiota and this raised the question whether those commensal bacteria would also produce neuromodulatory substances. In an attempt to address this question, bacteria from the intestines of our mice were isolated using Baird Parker Plates which are selective for Staphylococci. Colonies of these cultures were used to prepare supernatants under the same conditions that were used for other *S. aureus* strains. Regardless of incubation time however, these cultures did only grow up to an optical density of 4-5 which was significantly lower than the OD in experiments with JE2 (OD_{600} > 9). This is a concern because it is high population density that activates the production and secretion of neuromodulatory substances. Therefore, we cannot conclude the lack of neuromodulatory substances from our experiments using SSA from these bacteria. As shown in Fig S7, those SSA had only minor effects on spontaneous and distension-induced firing. It should be the aim of future studies to optimise culture conditions or test commensal strains isolated from mice to obtain a better understanding between neuromodulation and pathogenicity

3.4 Changes of membrane permeability are related to the inhibitory effect

Using the bacteriological approach, it emerges that SSA-induced neuromodulation is strongly dependent on the quantity of α-haemolysin as well as phenol-soluble modulins and potentially other pore-forming toxins in *S. aureus* strain Newman. These contribute to excitation and inhibition of spontaneous nerve activity and mechanosensitivity, respectively. Both of these substances are capable of interacting with the membrane of target cells. It is known that Hla monomers bind to the membrane via ADAM10 and oligomerise to pores with well-defined structural and physicochemical properties (Menestrina, 1986). The interaction of Psms and the membrane has been studied for the α-type (shorter) Psm δ-toxin and these studies suggest a less specific mode of action (Verdon et al., 2009). Thus far, it can only be assumed that Psmα1-4 and Psmβ1-2 act similarly. To investigate whether alterations of the cell membrane contributed to the effects of SSA on intestinal nerve activity, we isolated neurons from DRG and NG which innervate the intestine and studied effects of SSA on membrane integrity using the red-fluorescent dye propidium iodide (PI). PI is excluded from the

cytosol in cells with an intact membrane but can enter the cell after permeabilisation and possibly after pore formation. Previous studies (Blake et al., 2018; Chiu et al., 2013) have used calcium-sensitive dyes to investigate the effect of synthetic Hla and Psm on neuronal activity but this approach was not suitable for our study because these dyes can leach out from the cell when the cell membrane is perforated leading to a loss of fluorescence (preliminary data, not shown). In contrast, PI is sequestered in the cell by binding to the DNA (Fig 4A1).

SSA-JE2 increase PI fluorescence dose-dependently

To quantify changes of PI fluorescence as a surrogate for membrane permeability, we performed live imaging of PI fluorescence during application of SSA. When SSA-JE2 were applied to primary neuronal cultures, there was a profound change of PI fluorescence. While in primary cultures healthy neurons were largely PI-negative, SSA application increased intracellular fluorescence in these neurons indicating that components in SSA enabled membrane passage and DNA-binding of the PI dye (Fig 4A1 and 4A2). At high concentrations (5 %, 10 %), this change was observed in the majority of cells whereas less cells responded at lower concentrations. This is reflected in Fig 4A2 by a decrease of the overall Δfluorescence which was calculated from the SSA-induced increase of PI fluorescence of all selected cells (regions of interest) on the coverslip. We also noted that at lower concentrations of SSA-JE2 primary neurons did not respond as uniformly as it is shown for 10 % SSA-JE2 in Fig 4A2 suggesting that neurons differ in their susceptibility to SSA. To further investigate this variability, the response latency of responding neurons which were identified based on the SSA-induced increase of fluorescence (methods), was determined. Within the population of neurons responding to low concentrations of SSA, there was a remarkable variability of response latencies. Overall, response latencies decreased concentration-dependently (Fig 4A2, right) between 0.1 % and 5 % SSA-JE2. An increase of the concentration above 5 % had no further impact on the Δfluorescence but decreased the variability of the response latency (Fig 4A2, right). This indicates that response latencies may enable to detect smaller differences than Δfluorescence.

The observed variability of response latencies was reminiscent of the nerve recording data where pronounced differences between individual experiments were also observed. The data presented here indicates that this may be the result of a differential interaction of mediators in SSA with neurons/nerves with distinct properties. Subsequent experiments were designed to identify which mediators contributed to SSA-induced increase of PI fluorescence and investigate the possibility of a preferential targeting of particular populations of neurons.

Phenol-soluble modulins mediate SSA-induce increase of PI fluorescence

Our first aim was to establish which components in SSA mediated the increase of PI-fluorescence in order to relate these data to our nerve recording experiments. Therefore, SSA from different *S. aureus* mutants were applied to primary cultures of neurons from DRG and NG.

SSA were applied at a concentration of 10 % because it was expected that potential differences would be most pronounced at high concentrations. Application of 10 % SSA from the quorum sensing mutant (SSA-agrA$^-$) or SSA-Psm$^-$ did not increase PI-fluorescence significantly (Fig 4B1 for examples) indicating that agrA regulates the production of components that change membrane permeability and that it is Psms that predominantly contribute to this effect at a concentration of 10 %. In line with this, the lack of Hla (SSA-Hla$^-$) did result a response pattern that was not obviously distinguishable from the response to SSA-JE2 (Fig 4B1). The Δfluorescence per coverslip was similar to SSA-JE2 (Fig 4B2, left) which reflects that in both cases, the majority of cells responded to a concentration of 10 % SSA. This emphasises the dominating effect of Psm abundance and suggests that Hla might only mildly affect membrane permeability. To investigate whether Psms were potentially masking a contribution of Hla to SSA-induced changes of membrane permeability, SSA were applied at a lower concentration. As shown by the smaller Δfluorescence per coverslip (Fig 4B2, right), less primary neurons responded when SSA-Hla$^-$ were applied and additionally, responding cells also responded significantly later to SSA lacking Hla (figure 4B2, right) indicating that Hla does also affect membrane permeability.

Psms profoundly affected membrane permeability and contributed to inhibition of afferent firing in nerve recording experiments indicating that changes of membrane permeability are linked to inhibition. In addition to SSA lacking Psm, SSA from strain Newman also did not inhibit nerve activity and therefore, these SSA were tested in the PI live cell imaging. The overall response pattern to SSA-Newman (Fig 4B1, right) was similar to SSA-JE2 and the Δfluorescence (Fig 4B2, left) increased to the same extent suggesting that there are only small differences between these strains with regard to effects on membrane permeability. To test this hypothesis, response latencies were calculated for 5 % SSA-Newman. The response to SSA-Newman was significantly delayed compared to SSA-JE (Fig 4B2) but faster than the response SSA-Hla$^-$ which is consistent with Newman producing high amounts of Hla and Psms. The similarity between SSA-JE2 and SSA-Newman in this assay also indicates that the remarkable difference between those SSA in nerve recording experiments is not due to a profound difference of SSA-induced effects on membrane permeability.

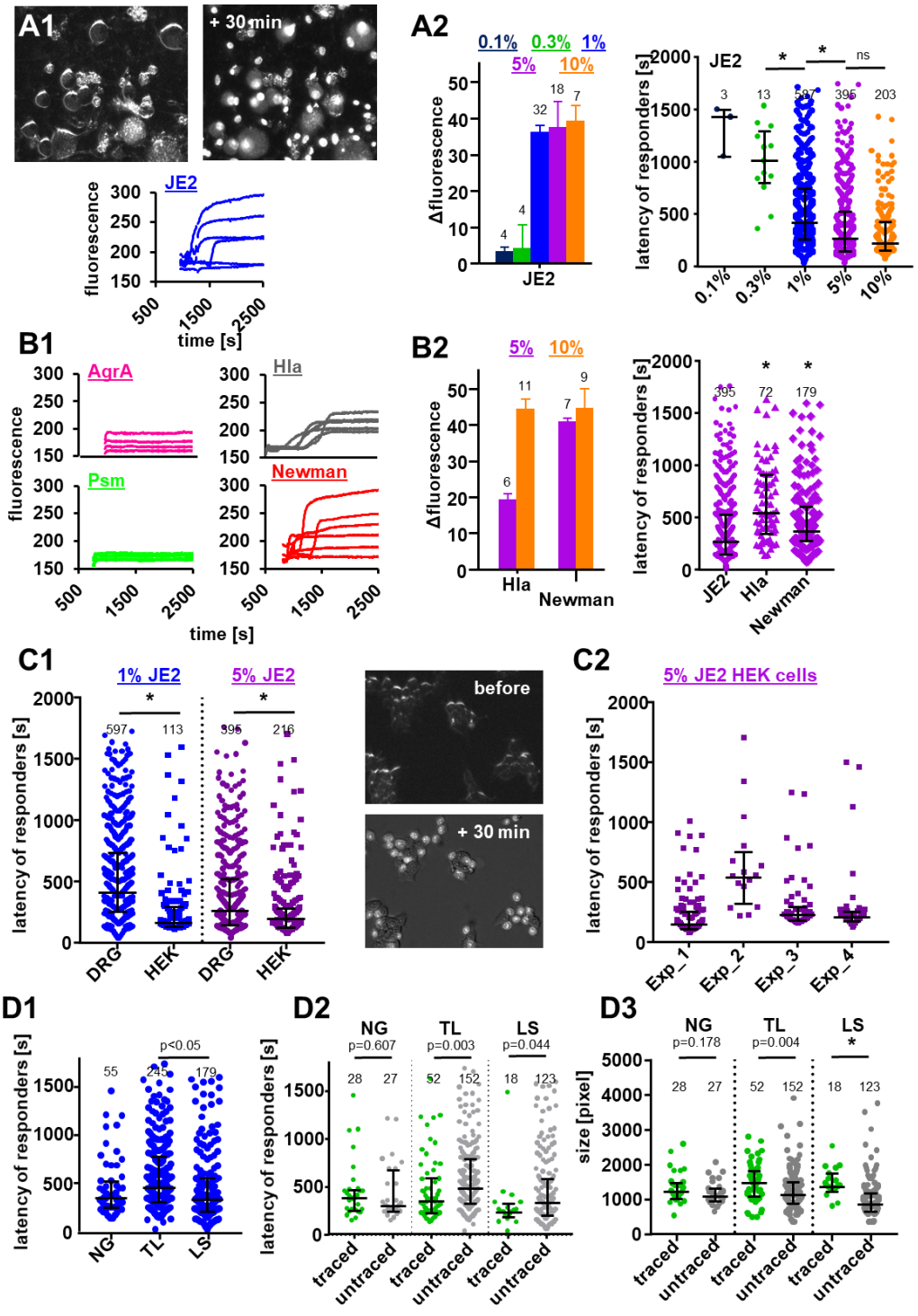

Figure 4: Effect of soluble mediators from SSA on cell membrane permeability

(A1) Example images showing DRG neurons after incubation with PI (left). SSA increase intracellular fluorescence during 30 min of application in neuronal and non-neuronal cells. Below the example image, a representative recording of intracellular fluorescence is shown for application of 10 % SSA-JE2. A fast increase of PI fluorescence can be seen. (A2) Quantification of SSA-JE2-induced changes of intracellular fluorescence. The change of fluorescence (left) per coverslip is a measure of the

number of responding cells and response latency (right) refers to the velocity of the response from responding cells. Δfluorescence increased and response latency decreased with increasing concentration.

(B1) Representative traces for experiments with 10 % SSA from different strains and mutants of S. aureus. SSA from the agrA and Psm mutant did not increase PI fluorescence whereas SSA-Hla⁻ and SSA-Newman did induce a similar response pattern compared to SSA-JE2. (B2) Application of 5 % SSA unveiled differences between SSA from the JE2 wildtype, the Hla-deficient JE2 and Newman with regard to the number of responding cells as indicated by the Δfluorescence (left) and the response latency (right). The response to SSA-Hla- was significantly delayed compared to SSA-JE2.

(C) Cell type affects susceptibility to SSA and bacterial factors contribute to the variability of response latencies. (C1) HEK293 cells responded faster to 1 % as well as 5 % SSA-JE2 and variability of the response latencies appeared smaller. Example images of HEK293 cells stained with propidium iodide before (top) or after (bottom) incubation with SSA-JE2. (C2) Some of the variability is attributable to the experimental conditions.

(D) Investigation of potential host-inherent factors contributing to the variability of response latencies to 1 % SSA-JE2 (D1) Comparison of response latencies between primary neurons isolated from the nodose, thoracolumbar or lumbosacral dorsal root ganglion. LS neurons had the shortest response latencies. (D2) Gut-innervating neurons of the different sensory ganglia were identified by ip injection of the green-fluorescent traced CTB-488. Visceral neurons from spinal ganglia had shorter response latencies. (D3) Spinal neurons identified by ip injection of CTB-488 were larger than untraced neurons.

For additional comparisons also refer to figure S8.

Non-parametric statistical analysis (Kruskall-Wallis Test with Dunn's Post-Hoc analysis or Mann-Whitney test); n numbers as indicated (coverslips or cells) obtained from 4-5 animals

Analysis of response variability: cell type, location of neurons within the spinal cord, peripheral projection and size

The pronounced variability of SSA-induced effects was a consistent finding in PI live cell imaging and nerve recording experiments. The heterogeneity of neurons in sensory ganglia could contribute to this variability. It has been found in RNA sequencing studies that up to 16 different neuronal subtypes are present in individual sensory ganglia and that those are characterised by the expression specific of receptors, neurotransmitters and membrane glycolipids (Li et al., 2016; Usoskin et al., 2014).

Cell type. To test whether this was indeed the case, we performed PI live imaging experiments on cells with a higher degree of uniformity. The HEK293 cell line is of human origin and is widely used in research because of the easiness of genetic manipulation. Application of SSA in different concentrations and from different bacteria largely confirmed our findings from primary neurons with regard to concentration-dependency and bacterial components contributing to the increase of PI fluorescence (data not shown). Compared to primary neurons, HEK293 cells had shorter response latencies at all tested concentrations (Fig 4C1 for 1 % and 5 % SSA-JE2) which may reflect the higher susceptibility of cells with human compared to murine origin and differences between primary cells compared to cell lines (Strobel et al., 2016).

The response latencies of HEK293 still displayed some degree of variability albeit it was smaller than the variability in primary neurons which is consistent with HEK293 cells being a homogeneous

population (Fig 4C1, not shown for experiments with other mutants). It does however indicate that factors other than neuronal heterogeneity contribute to the variability and we compared the data from individual experiments to investigate this further (Fig 4C2). We found that the response latencies in 1/4 experiments was significantly higher indicating that the composition of the SSA-JE2 varied between experiments because other factors such as temperature, pH etc. were tightly controlled. Thus, bacterial variability partially contributes to overall variability of response latencies.

Location of sensory ganglia. The higher uniformity of the response latencies in the HEK293 cell line suggested that inherent factors of primary neurons may predispose for susceptibility to SSA. The distal small intestine is innervated by sensory neurons from the nodose ganglion (NG) as well as thoracolumbar (TL) and lumbosacral (LS) DRG. These are known to transmit different aspects of the intestinal sensation to the brain and therefore, the response latencies of neurons from these ganglia were compared. When 1 % SSA-JE2 was applied, the response of TL neurons was delayed compared to LS neurons, indicating that it is the lumbosacral neurons which are known to be activated by stimuli of low intensity, that are particularly susceptible to SSA (Fig 4D1). In line with this, the response latency of NG neurons also tended to be lower compared to TL neurons but this was not significant. Overall, the response latencies of neurons from all locations still displayed a large degree of variability indicating that it is not primarily the location of sensory ganglia that predisposes for a higher susceptibility of neurons to SSA.

Peripheral projection. Individual sensory ganglia comprise neurons with peripheral projections to the skin, the lungs, the heart, the digestive tract etc. We were particularly interested to investigate the effect of SSA on neurons with intestinal projections which are known to have a distinct expression profile compared to neurons innervating other organs (Peeters et al., 2006b). To identify the 3-5 % of neurons in NG, TL and LS that innervate the intestine, CTB-488 was injected intraperitoneally and PI live imaging was undertaken on retrogradelly labelled neurones (Fig 5B1). In line with our findings from whole ganglia neurons, traced LS neurons were also most susceptible to SSA compared to traced neurons from other sensory ganglia (Fig S8, $median_{LS} = 237.1$ s vs $median_{TL} = 355$ s vs $median_{NG} = 380$ s, $p < 0.05$, Kruskal-Wallis test). Additionally, labelled LS and TL neurons had significantly shorter response latencies than unlabelled neurons (figure 4D2). This suggests that inherent properties of intestine-innervating neurons which constitute the majority of traced TL and LS cells after ip CTB injection, increases the susceptibility of these neurons for SSA. In contrast, a larger proportion of traced neurons from the NG are likely to innervate other visceral organs. The response latencies between traced and untraced neurons from the NG were not significantly different and this

supports the hypothesis that it is non-intestinal (visceral) neurons that are less sensitive to SSA-induced changes of membrane permeability. Interestingly, the variability of response latencies was smaller in traced neurons than in untraced neurons which is consistent with being a more homogeneous population.

Size. In addition to the peripheral projection, neurons within sensory ganglia have been distinguished based on their cell size. Because cell size is also linked to the function of the sensory neuron, it was particularly interesting to assess whether size was correlated with sensitivity SSA. When we compared the cell size of responding and non-responding neurons, we found that the former were significantly larger than the latter ($median_{resp}$ = 1093 px vs $median_{non\text{-}resp}$ = 810 px, $p < 0.05$, Mann-Whitney test). A similar trend was observed for all locations of sensory ganglia and regardless of whether or not neurons were labelled by ip injection of CTB-488 (Fig S8B) indicating that it is the larger cells that were more susceptible to SSA.

Because traced neurons responded earlier than untraced ones (Fig 4D2), the sizes of these cells were compared. Traced neurons from spinal sensory ganglia (TL and LS) were significantly larger than untraced neurons (Fig 4D3). In contrast, traced and untraced NG neurons had similar response latencies and cell sizes were not significantly different (Fig 4D3). We further investigated the effect of cell size on response latencies and found a significant inverse correlation between these variables when 1 % SSA-JE2 was applied (Fig S8D1). In addition, there was a significant difference of average response latencies between the smallest and the largest traced neurons (Fig S8D2). Together, these results are in support of the hypothesis that cell size contributes to the response variability of primary sensory neurons. The finding that neurons labelled after ip infection of CTB-488 (visceral neurons) were larger than untraced neurons requires further investigations.

Overall, the results presented above indicate that cell size and peripheral projection contribute most the variability of the neuronal PI responses to SSA. None of these factors however, clearly separated responding and non-responding neurons which suggests that the presence or absence of specific mediators has a predominant effect in PI live imaging experiments.

Changes of membrane permeability can ultimately lead to cell death and PI accumulation in the nucleus is also used to assess cell viability. Therefore, we quantified cell death manually (methods) to establish whether PI could be used to distinguish both events. In response to SSA application, intracellular PI fluorescence and cell morphology changed remarkably in some primary neurons. The proportion of these neurons increased dependent on SSA-JE2 concentration which is reflected by a

decrease of intact non-fluorescent neurons (Fig S9A1). At a concentration of 20 %, a large decrease of the number of alive cells was observed within the first 10 min whereas at lower concentrations (10 % and 5 % SSA), the onset of this decrease was delayed and the degree significantly smaller (Fig S9A2). This constitutes a remarkable difference between manual analysis and PI live imaging. While 5 % SSA-JE2 had only small effects on cell viability, it induced a fast response in PI live imaging and this suggests that the higher sensitivity of the PI live imaging can indeed be used to detect changes of membrane permeability prior to cell death.

We also tested for potential strain and size differences using this assay. SSA-Newman application delayed the onset but not overall degree of cell death compared to SSA-JE2 (Fig S9A2). This indicates that SSA-Newman have a reduced cytotoxicity which is in line with the delayed response of primary neurons to SSA-Newman in the PI live imaging. The cell size of dead cells was also significantly larger than cells that were not killed by 20 % SSA-JE2 (Fig S9B, right, $median_{dead}$ = 2895 px vs $median_{alive}$ = 2298 px, $p < 0.05$, Mann-Whithney test). This difference ($\Delta area = 600$ px) which corresponds to 5-6 pixel (3 µm) with regard to the diameter is however small compared to published data on cell sizes of DRG neurons and suggests that it neurons within the same population that is affected differently by SSA. The diameters were 24.5 and 21.5 µm suggesting that it is small/medium-sized neurons that respond to SSA.

These cell-based experiments strongly support that Psms and Hla importantly contribute to the effects of SSA on visceral sensitivity. These substances are known to bind and activate formyl peptide receptors (FPRs) and A Disintergrin And Metalloprotease 10 (ADAM10), respectively. FPR and ADAM10 (Inoshima et al., 2011, Schreiner et al., 2013) activation both induce intracellular signalling pathways which could be involved in the effects of SSA. Binding of α-haemolysin monomers to ADAM10 is also a prerequisite for pore formation.

3.5 Receptors for virulence factors are expressed in neurons and the intestine

The possibility that Psms and Hla induce receptor-mediated effects on primary neurons and intestinal tissue was explored by investigating the expression of FPRs and ADAM10 respectively. It has been shown that these are indeed present in dorsal root ganglia neurons but we wanted to extend these findings and compare the abundance between different subpopulations of sensory ganglia and in the intestine. We also analysed the expression of sphingomyelin synthesase 1 (Sgms1) because sphingomyelin is associated with the cell's susceptibility for Hla (Virreira Winter et al., 2016).

Quantitative reverse transcription PCR using TaqMan assays confirmed the expression of our target genes in whole DRG and the intestine (Fig 5A). In all samples, ADAM10 was the most abundant gene followed by Sgms1 and FPRs. All genes except for ADAM10 were significantly higher expressed in whole DRG compared to intestinal tissue. This difference was particularly pronounced for the FPRs whose expression in DRG was about one log unit above the intestinal expression (Fig 5A) suggesting that FPR-mediated effects of Psms would predominantly be exerted through afferent neurons. Next, we compared the expression level of our genes of interest between different sensory ganglia. NG and lumbosacral DRG which comprise higher proportions of parasympathetic neurons were found to express larger quantities of FPR2 and FPR3 compared to cervicothoracic and thoracolumbar DRG (Fig 5B2) which may relate to the relatively higher susceptibility of neurons from these ganglia to SSA. All the other transcripts were equally abundant in all sensory ganglia.

Ultimately, the expression of ADAM10, Sgms1 and FPRs was assessed in RNA from 20 visceral neurons identified by fluorescence after ip injection of CTB-488 (Fig 5B1). In these samples, ADAM10 and Sgms1 but not FPRs were expressed at levels above the detection threshold. ADAM10 expression relative to housekeeping genes was significantly lower in traced cells from the NG and LS DRG than in whole DRG ganglia from the respective location (Fig 5B3) suggesting that cells other than visceral neurons may contribute to the higher expression level of ADAM10 in whole ganglia. With regard to Sgms1, it was equally expressed in visceral neurons and whole ganglia in all sensory ganglia. The low abundance of FPRs in RNA from traced neurons suggests that these cells either do not express high levels of FPRs or FPRs are not particularly enriched in this population.

Overall, we find that the receptors for the main neuromodulatory components of SSA are expressed in sensory ganglia and this suggests that receptor-mediated effects may contribute to the observed changes of nerve activity and SSA-induced changes of membrane permeability.

Neuronal expression of genes of interest was high which is consistent with the idea that sensory neurons are specialised to detect bacterial compounds and that detection of these mediators contributes to neuronal symptoms during gastroenteritis including pain, nausea and vomiting. Albeit their abundance was lower than in sensory ganglia, transcripts of all genes were also found in intestinal tissue which raised questions with regard to their expression pattern in different intestinal cell types and the effects of SSA components on intestinal function.

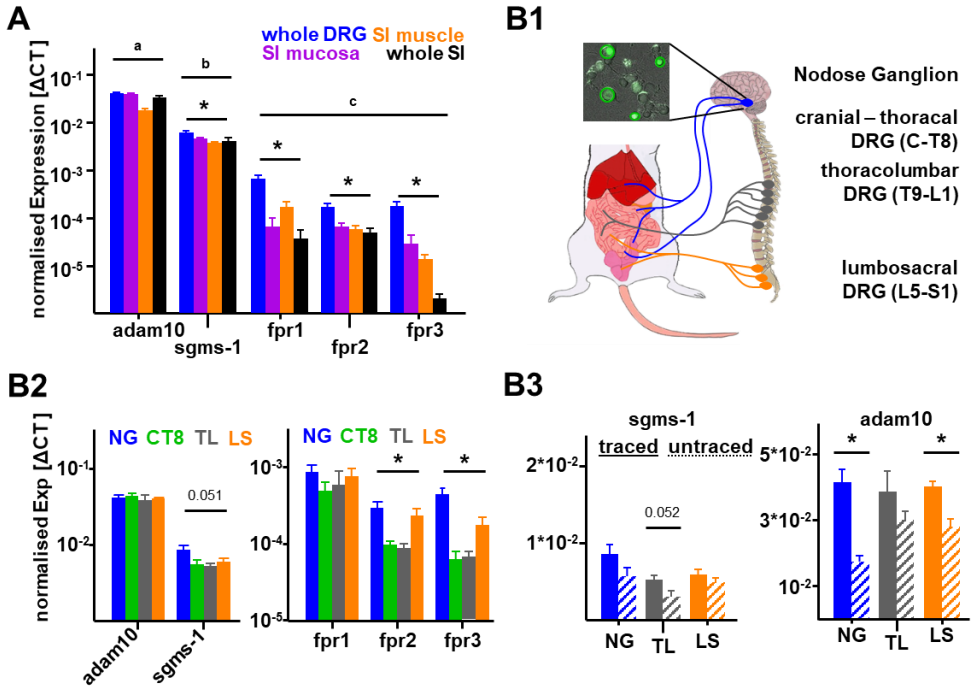

Figure 5: Putative receptors for Hla and Psms are expressed in DRG, NG and the small intestine.

(A) Among the receptors, adam10 was most abundant in all tissues followed by sgms-1 and the fprs. Those were more highly expressed in DRG compared to intestinal tissue.

(B1) Schematic showing the spinal location of DRG with high proportions of intestine innervating neurons. Receptor expression was compared between pooled DRG from the different levels of the spinal cord and the NG. In subsequent experiments, visceral neurons were isolated from primary cultures of DRG and NG based on their green fluorescence after ip injection of CTB-488. (B2) adam10, sgms-1 and fpr-1 were equally expressed in NG and DRG. Expression of fpr2 and fpr3 was higher in NG and LS DRG compared to cranial and TL DRG. (B2) We did not find a difference of sgms-1 expression between whole ganglia and 20 gut-innervating neurons from the NG or spinal cord. Normalised Adam10 expression was lower in traced neurons compared to whole ganglia. The expression of fprs in 20 traced neurons was below the detection limit.

Parametric statistical analysis (ANOVA with corrected Post-Hoc analysis); N = 3-4 animals per data point, technical duplicates

3.6 Strain and mutant specific effects on epithelial secretion and motility

Others have previously described the expression of adam10 and fprs in mouse intestine and investigated the function of ADAM10 and FPRs in the epithelium (Babbin et al., 2007; Chen et al., 2013b; Tsai et al., 2014). Our findings suggest that neuronal expression of these proteins is important for the detection bacterial compounds. The majority of neurons in the intestine is part of the ENS which regulates intestinal functions largely independent of the CNS. Because diarrhoea is a common symptom during bacterial gastroenteritis, we were interested to test the effect of SSA on intestinal secretion and used Ussing Chamber experiments to record short circuit currents (SCC) as a readout of intestinal permeability and secretion.

Serosal application of 10 % SSA-JE2 to mucosa-submucosa preparations of mouse small intestine induced a profound increase of SCC. The response profile was characterised by a steep increase of SCC which plateaued by 1000-1500 s of application (figure 6A1). At lower concentrations (1 %, data not shown), the overall increase (AUC) as well as the slope of the response within the first 700 s was significantly smaller than at 10 % SSA-JE2 indicating that the interaction of bacterial substances with intestinal cells in mouse has a prosecretory effect.

In the present study, we were particularly interested to investigate which mediators contributed most to the SSA-induced increase of SCC and therefore applied SSA from different *S. aureus* to the serosal chamber. Application of SSA from *S. aureus* other than JE2 increased SCC to different extents (Fig 6A1). The prosecretory effect of SSA was profoundly reduced when SSA from the quorum sensing mutant (SSA-SgrA$^-$) were applied (Fig 6A1, pink trace). This is shown by a smaller overall increase of SCC (AUC, Fig 6A2, left) as well as a smaller time-dependent increase immediately after application (slope, Fig 6A2, right) and indicates that it is virulence factors that mediate the increase of SCC. SSA lacking either Hla or Psm were applied because it was these components that were mainly involved in SSA's effects on nerve activity and membrane permeability. The SCC response profiles indicated a reduction of the prosecretory effect of SSA-JE2 when they lacked Hla- or Psm-mutant (Fig 6A1, gray and green traces for SSA-Hla$^-$ and SSA-Psm$^-$ respectively). In the statistical analysis however, only SSA-Hla$^-$ had a significantly smaller effect on the slope of the early response and tended to reduce the AUC (Fig 6A2, left). In contrast, neither of these variables was significantly different between SSA-JE2 and SSA-Psm which this suggests that it is Hla that plays a major role SSA-induced secretion.

The effect of supernatants from Newman strain of *S. aureus* on SCC was also tested because of the remarkable difference between SSA-JE and -Newman in nerve recording experiments. With regard to their prosecretory effect, 10 % SSA-Newman appeared to delay the SSA-induced increase of SCC compared to SSA-JE2 (Fig 6A1, red for SSA-Newman) but the magnitude of the overall secretion (AUC, Fig 6A2, left) and the slope of the response (Fig 6A2, right) were not significantly different between SSA-Newman and SSA-JE2. This indicates that mediators such as Hla that are equally produced by both strains contribute to the prosecretory effect of SSA and therefore, these findings are consistent with the mutant analysis. It also suggests that the lower abundance of other components in SSA-Newman (Fig S6) may possibly reduce their capacity to increase secretion from intestinal epithelial cells in some preparations.

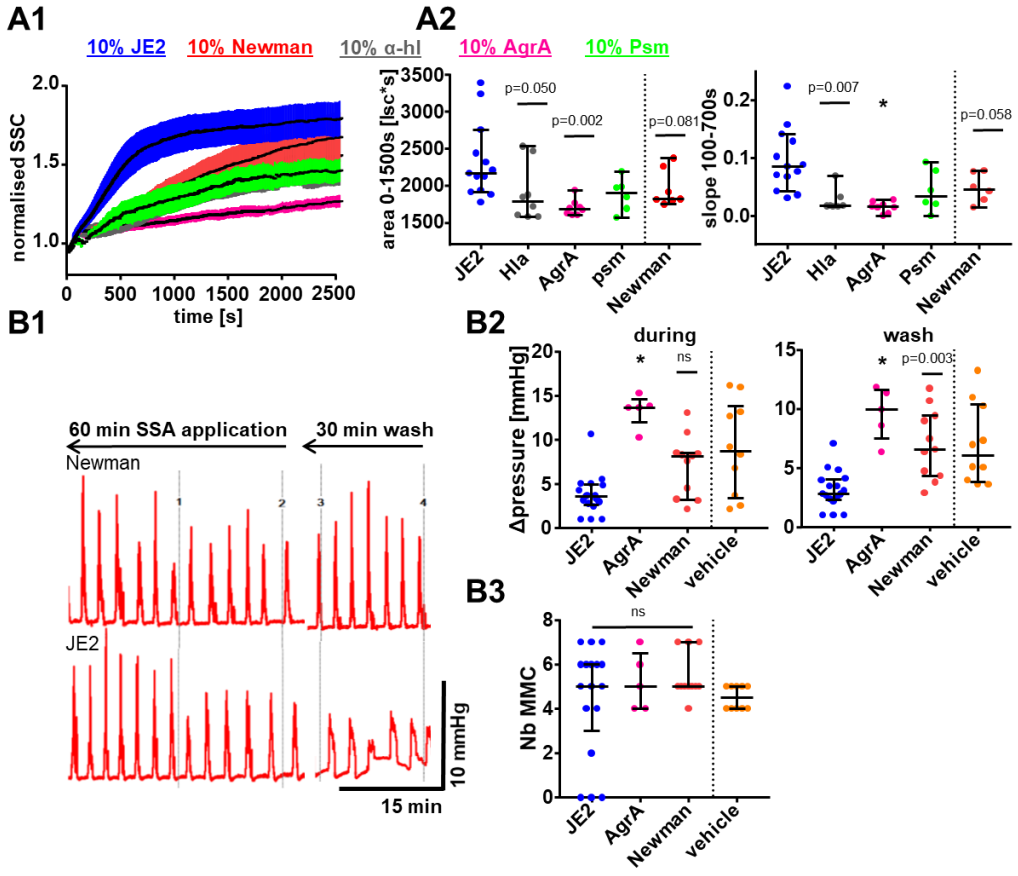

Figure 6: Soluble mediators in SSA-JE2 affect intestinal function.

(A1) Aligned overlay of SCC measurements (mean ± SEM) for different mutants and strains of S. aureus. (A2) The SSA-induced increase of AUC was significantly smaller in the AgrA mutant compared to wild type JE2 (left). AgrA- as well as Hla-deficient SSA reduced slope of the SSA-induced increase SCC (right). SSA-JE2 and Newman were not statistically different (Mann-Whitney test); N (JE2) = 13, N(Hla) = 8, N(AgrA) = 8, N(Psm) = 6 from seven animals

(B1) Example traces for motility measurements were SSA were applied for 60 min directly after inducing MMC in the colon followed by a 30 min wash-out period. Contraction amplitude and number were determined in the 15 min interval at the end of the incubation period. (B2) The SSA-JE2-induced decrease of contraction amplitude was reverted when the quorum sensing regulator AgrA was mutated. SSA-Newman had a smaller inhibitory activity in the wash period. (B3) The number of MMC was not statistically different between JE2 and the other supernatants

N(Newman) = 11, N(AgrA) = 5; Vehicle (N = 8) and JE2 (N = 17) were reproduced from chapter II (Figure 4, p 77)

The data presented here argue for a Hla-dominated prosecretory effect of SSA and this is in contrast to the Psm-dependence of SSA-induced increase of PI fluorescence in primary DRG and NG neurons. It suggests that modulation of enteric neuron activity may not be the primary mechanism underlying the increase of SCC during application of SSA. Indeed, in our previous study, we reported that blockage of neuronal activity and synaptic transmission did not reduce the SSA-induced secretion.

Regulation of gastrointestinal motility is another major function of the ENS. Disturbed bowel movements are frequently experienced by patients with food poisoning or intestinal infections suggesting that bacteria-induced changes of neuronal activity could potentially be involved in gastrointestinal dysmobility. To test this hypothesis, the effect of SSA on colonic migrating motor complexes (MMC) was investigated using an established ex-vivo motility assay (Keating et al., 2010). Previously, we tested the effect 5 % bath-applied SSA-JE2 on MMC patterns and refer to this data to evaluate potential differences between JE2 and other *S. aureus* bacteria (Uhlig, this thesis).

As can be seen in Fig 6B3, the frequency of contractions was not significantly different between the supernatants from different *S. aureus* (SSA-JE2, SSA-Newman and SSA-agrA$^-$) and was also similar to the frequency of contractions during vehicle application. In fact, the number of 5 MMC in 15 min has also been reported in other studies (Keating et al., 2010) which suggests that neither of the supernatants or vehicle interferes with the generation of MMC.

In sharp contrast, SSA profoundly altered contraction amplitude. Fig 6B1 (bottom) shows the remarkable decrease of MMC amplitude during prolonged application of SSA-JE2. This decrease was significantly different from vehicle application (Fig 6B2, vehicle and JE2 reproduced from Chapter II, Figure 4)and continued throughout the wash-out period. In the presence of SSA-Newman, the amplitude of contractions also decreased over time (Fig 6B1, top) but overall, the average contraction amplitude ($median_{SSA\text{-}Newman}$ = 8.4 mmHg, N = 11) tended to be higher than during SSA-JE2 application ($median_{SSA\text{-}JE2}$ = 3.6 mmHg, N = 17) and close to the amplitude of contractions during vehicle application ($median_{vehicle}$ = 8.8 mmHg, N = 10) suggesting a reduced inhibitory effect of SSA-Newman. Indeed, there was a significant difference of MMC amplitude between SSA-Newman and SSA-JE2 during the wash-period because contraction amplitude after SSA-Newman application did not decrease further (Fig 6B2, right). This was in contrast to SSA-JE2 and indicates that the abundance or activity of MMC-inhibiting mediators in SSA-Newman is lower compared to SSA-JE2.

The neuromodulatory and prosecretory effects of SSA were largely dependent on AgrA-regulated virulence factors and therefore, it was tested whether they also contributed to SSA-induced inhibition of motility. The contractions that were generated in the presence of SSA-AgrA$^-$ had an amplitude of 13.6 mmHg (median, N = 5) which was significantly higher than during JE2-application and also tended to be higher compared to contractions during vehicle perfusion (Fig 6B2, left). While the latter may be attributable to the presence of bacterial compounds such as components of the bacterial cell

that are not regulated by AgrA, the former strongly supports the idea that it is AgrA-regulated virulence factors that inhibit contraction amplitude.

In summary, these experiments are consistent with *S. aureus* quorum sensing-regulated molecules affecting intestinal function. We find that Hla predominantly contributes to the increase of intestinal secretion and hypothesise that it is factors in addition to Hla and Psms that affect MMC amplitude because SSA-Newman had a smaller inhibitory effect.

4 Discussion

In the present study, we have used a microbiological approach to identify bacterial compounds with neuromodulatory effects. Two classes of heat-labile membrane-interacting toxins, α-haemolysin and phenol-soluble modulins were found to contribute to excitation and inhibition of intestinal afferent firing. Those are produced by *S. aureus* at high population density dependent on the quorum sensing activated gene regulator agrA and also modulate epithelial secretion and motility.

4.1 Bacteriological approach to investigate host-pathogen interaction

In order to study a direct interaction between bacteria and neurons, we used supernatants of bacterial cultures with well-described genotypes containing a number of secreted virulence factors. Others have applied supernatants from mucosal biopsies and faecal samples to isolated neurons and intestinal nerves to investigate mechanisms underlying changes of visceral sensitivity (Sessenwein et al. 2017; Valdez-Morales et al. 2013; Ibeakanma et al. 2011) and have identified a role for both host and bacteria derived mediators (Ibeakanma and Vanner 2010; Valdez-Morales et al. 2013), respectively. In contrast, investigation of these mediators individually (Dinic et al., 2018; Ochoa-Cortes et al., 2010) in a more targeted approach provides a more mechanistic analysis but may underestimate their effects because there may be synergistic interactions between a number of factors secreted simultaneously. In this study we have used supernatants from a number of different strains of *S. aureus* to "dissect out" the mediators that contribute to modulation of intestinal sensory function.

4.2 Neuromodulatory substances in *S. aureus* supernatants

Using SSA-JE2 as our wild-type strain we found a dramatic effect on intestinal afferent firing with an early period of excitation lasting about 30 min followed by a profound inhibition that in some experiments resulted in complete abolition of afferent firing and response to distension. We first tried to determine if this biphasic response was mediated by separate classes of mediators causing excitation or inhibition.

***Phenol-soluble modulins* (*Psm*)**

Using a mutant *S. aureus* missing Psms, we found that the excitatory phase was unaffected while the inhibition of spontaneous and mechanosensitive firing was markedly attenuated. This is consistent with our hypothesis that different mediators are responsible for excitation and inhibition. This effect

was mirrored in isolated DRGs in which the SSA-induced membrane permeabilisation as measured by the rapid increase of PI fluorescence was markedly attenuated using SSA-Psm^{-}. This suggests a major role for Psms in bacteria-induced modulation of afferent sensitivity. Psms are a group of short amphipathic peptides highly expressed by pathogenic *S. aureus* (Wang et al., 2007) and have been shown to effectively lyse human neutrophils, erythrocytes and T cells (Laabei et al., 2014; Wang et al., 2007). Others have found that Psm-α3 induces calcium transients and action potential firing in cultured neurons (Blake et al., 2018) which might imply an excitatory rather than inhibitory effect. Psms have also been shown to increase intracellular calcium via interaction with the formyl peptide receptor (Rautenberg et al., 2011) again consistent with an excitatory effect. We and others have found that FPRs are expressed in sensory ganglia and they were also detected FPRs in some colonic neurons (Hockley et al., 2018). It is possible that the excitatory effect leads to a longer-term excitatotoxicity and inhibition of firing. However, since in our study excitation persists in the absence of inhibition, these are not linked. In contrast, it may be that receptors other than FPRs are involved in Psm-mediated nerve inhibition. Their structural similarity to β-defensins and other ligands of the human mas-related g-protein-coupled receptor MRGPRX2 (Bader et al., 2014) suggests that Psms may be involved in itch sensation either directly or following mast cell degranulation (Nakamura et al., 2013).

***α-haemolysin* (*Hla*)**

In contrast to the prominent role of Psms in inhibition, we found that Hla contributes to excitation of intestinal afferents. Thus, SSA from the Hla mutant of SA failed to excite afferent firing but instead caused a monophasic inhibition. Moreover, the Hla-deficient supernatants continued to increase PI in DRG neurones albeit at a longer latency to that of wild-type supernatant. These observations are consistent with previous studies showing that Hla can induce action potential firing and calcium transients in primary sensory neurons (Chiu et al., 2013). Our observation also suggest that it is ion influx through pores formed by Hla that directly increases neuronal excitability. Given its pore forming capacity, it is intriguing that Hla in contrast to Psms did not contribute to inhibition of intestinal nerve activity and this indicates that Hla-induced pores are less disruptive for the cell membrane. Indeed, the lack of Hla only marginally reduced the ability of SSA to increase intracellular PI fluorescence in our live imaging experiments and Hla-pores have a defined structure that allows the passage of ions (Ca^{2+}, K^{+}) and nucleotides (Menestrina, 1986; Vandenesch et al., 2012a). Our finding that ADAM10 is expressed in sensory neurons suggests a possible mechanism of action of Hla. ADAM10 is a membrane bound metalloprotease that is implicated in Hla pore formation. ADAM10 is required for Hla binding and also needed for Hla-mediated cytotoxicity.

In addition to Hla, other pore forming toxins such as Pvl could also be contribute to the excitation caused by SSA. We found that Pvl-deficient SSA tended to induce attenuated excitation compared to wildtype ($p > 0.05$). Blake et al. (2018) report that the two-component leucocidin HlgAB can activate DRG neurons contributing to pain signalling. Our data suggest that multiple mechanisms contribute to pore formation and may have redundant and synergistic functions (Los et al., 2013). In both this and the previous studies however, it was Hla that played the major role in increased intestinal afferent firing and spontaneous pain during *S. aureus* infection respectively which may imply that the large excitatory response in our study directly reflects a nociceptive signal.

Proteases

Recent studies have shown an inhibitory effect of commensal strains of bacteria on DRG excitability which may contribute to their pain-regulating effects and was mediated by serine proteases acting on PAR4 receptors (Sessenwein et al., 2017). In our microbiological approach, we used strains of SA devoid of bacterial proteases to investigate protease involvement in intestinal afferent signalling. However, we found that SSA from these bacteria continued to generate a biphasic afferent response with excitation followed by inhibition. The inhibitory effect was attenuated but this was not significantly different from wildtype. However, in about half of the experiments the inhibitory effect was absent using the protease mutant. This variability is inherent in studies of bacteria-host interaction because of the marked effect of population density and environmental factors in the secretion of virulence factors (Blake et al., 2017). Thus, bacterial proteases may potentially directly contribute to sensory signalling but, because bacterial proteases are important virulence factors for tissue invasion, it is possible that, in-vivo, they also facilitate the penetration of neuromodulatory components to nerve terminals situated beneath the mucosal epithelium.

4.3 Relevance to strain pathogenicity

The speed at which bacteria can mutate constitutes a major difficulty in antibiotic treatments, particularly in SA-associated diseases (Kennedy et al., 2008). Acquisition and loss of genes generates numerous strains and consistent with this genetic variability, we found that SSA from three strains of SA had differing effects on intestinal afferent nerves. In line with published analyses of bacterial secretomes (Chapman et al., 2017a) and our mutant analysis, the Newman and JE2 strain which produce large amounts of Hla, both profoundly increased intestinal afferent firing. Newman, in

contrast to JE2, did not inhibit nerve activity during prolonged SSA application and this could be due to the absence of Pvl and smaller amounts of proteases. Pvl-induced IL-1β secretion was shown to be potentiated by Psms suggesting a synergistic interaction between Pvl and Psms (Perret et al., 2012). Proteases may enable tissue penetration of inhibitory substances in the JE2 strain but less so in Newman strain. The SH1000 strain displayed an intermediate phenotype with regard to excitation and inhibition which matches its genotype (Horsburgh et al., 2002) and pathogenic potential in a tail infection model compared to other strains of SA (Thänert et al., 2017). These findings suggest that neuromodulation is an important component of host-pathogen interaction and future studies should address whether the observed strain differences also translate into measurable physiological parameters such as immune activation.

4.4 Relevance during infection

SSA-induced modulation of the gut-brain axis will have consequences for GI symptoms such as discomfort, pain and fullness. In addition, bacterial mediators may also induce important reflex functions including secretion, motility and neuro-immune interactions which may depend upon the effect of SSA on other targets within the gut wall including actions on enteric neurones, smooth muscle cells and epithelium.

In terms of secretion, serosal application of SSA-JE2 caused a profound increase of transepithelial ion movements as revealed by changes in short-circuit current. Like the neuromodulatory effects described above, this too was dependent upon quorum sensing-regulated molecules. However, only Hla- but not Psm-deficient SSA significantly reduced SSA-induced secretion which suggests that it is either the activation of secretomotorneurons by specific pore-formation or the activity of ADAM10 that contributes to the prosecretory effect of SSA. We previously found that modulation of neuronal activity is not involved in SSA-induced secretion since it persists after treatment with tetrodotoxin (Uhlig, unpublished data) implicating a direct effect on the epithelium. In this respect, Hla has been shown to induce epithelial damage via ADAM10-mediated cleavage of cell-adhesion proteins such as E-Cadherin (von Hoven et al., 2016) primarily contributes to the severity of SA lung and skin infections (Becker et al., 2014).

Virulence factors in SSA-JE2 were also found to modulate colonic contractile activity. Wildtype SSA caused inhibition of migrating motor complexes. This degree of inhibition was attenuated using the

Newman strain. Since both strains produce Hla and Psm this differential effect on motility may implicate other bacterial mediators. As the Newman and JE2 strains also had different effects on afferent nerve activity, it is possible that the lower amounts of proteases or the lack of Pvl in the Newman strain also reduces its inhibitory effect on smooth muscle function. Interestingly, commensal and probiotic bacteria can also inhibit contractions (Gong et al., 2017; Guarino et al., 2008; Massi et al., 2006) and this is potentially mediated by modulation of myenteric neuron activity by cell wall glycolipids (Mao et al., 2013).

Overall, our findings suggest that a direct interaction between neurons and bacterial mediators is involved in sensory symptoms during bacterial infections whereas an interaction of those components with epithelial and smooth muscle cells may underlie intestinal symptoms such as diarrhoea and dysmotility. Future studies should also address whether intestinal immune cells are involved in SSA-induced effects. SA is known to induce cytokine secretion from neutrophils and the release of mast cell mediators, both of which are present in the gastrointestinal tract.

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6 Supplementary Figures

Fig S1

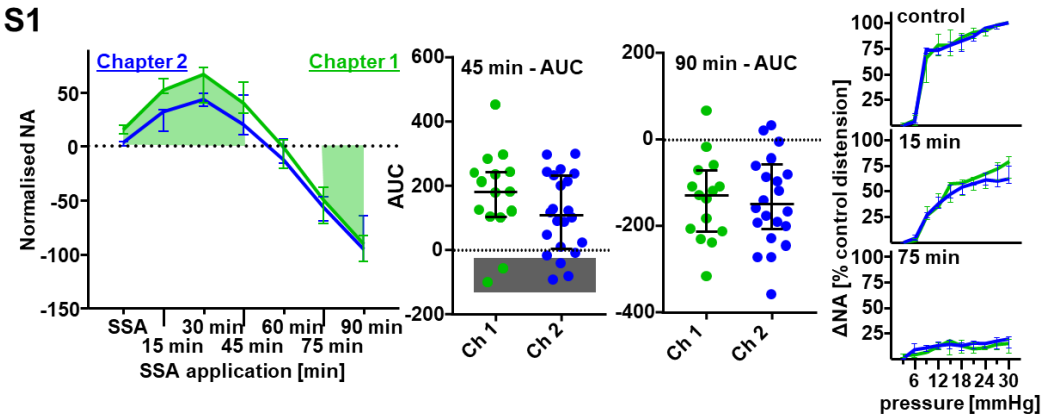


Figure 7(S1): Comparison of SSA-(20 % v/v)-induced changes of nerve activity and mechanosensitivity between our current and previous study.

(A) Response profile of nerve activity indicates that SSA induce excitation until to 45 min of application and inhibition after 75 min until the end of the experiment.

(B) The integrated excitatory activity (left) and inhibitory activity (right) were not significantly different between both studies. Excitation tended to be reduced in the current study and in some experiments (gray background) nerve activity did not increase above vehicle-induced excitation.

(C) The effect of SSA on mechanosensitivity in the current study was comparable to the alteration of distension-induced firing by SSA our previous study.

Fig S2

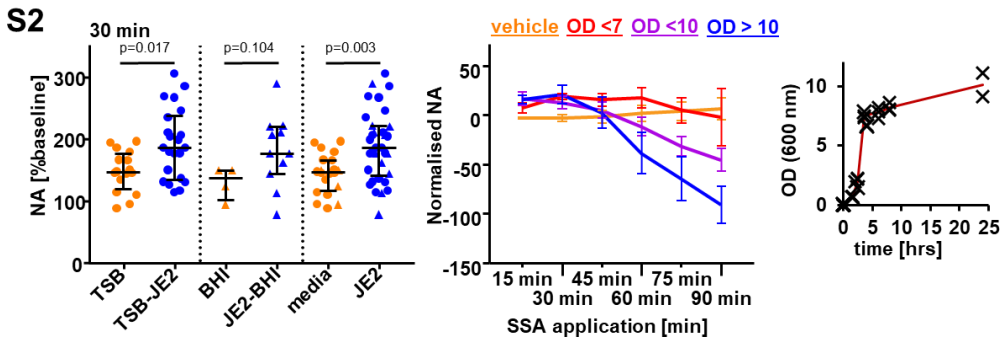


Figure 8(S2): Effect of growth stage on nerve response profile to SSA

(A) Comparison of the excitatory effect of SSA-JE2 produced in two different bacterial growth medias. SSA prepared in TSB significantly increased nerve activity (NA) relative to vehicle. The difference between brain heart infusion buffer (BHI) and SSA-JE2 prepared in this growth medium was not significantly different. The pairwise comparisons between TSB and BHI as well as JE2-TSB and JE2-BHI was not significantly different.

(B) Data presented in Figure 1(p 117) was re-analysed by optical density of SSA rather than time of incubation. SSA with high optical density decreased nerve activity during long-term application. This inhibition was reduced when SSA with lower optical density were applied. In these experiments, the excitatory effect of SSA was alleviated compared to our previous data.

(B) Correlation of optical density and time of incubation. After a lag phase, bacteria enter exponential growth stage by about 3 hours and during that time optical density increases rapidly from OD = 2 to OD = 7. Longer incubation reduces the growth rate (increase of OD) and optical density plateaus. This is referred to as stationary phase.

Fig S3

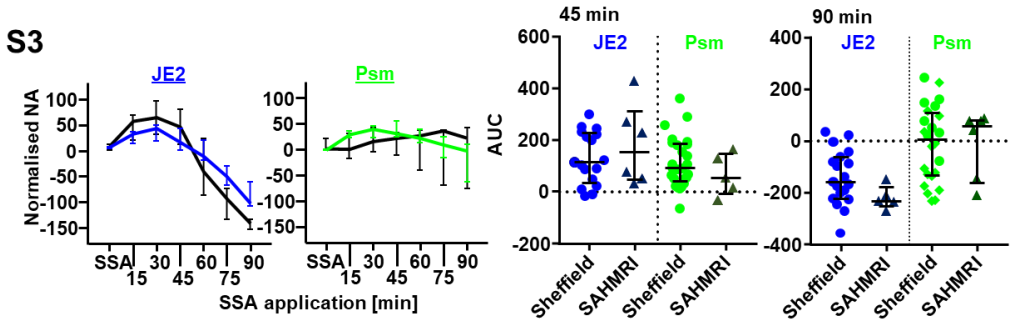


Figure 9(S3): Comparison of the effect of SSA from two datasets obtained from different cohorts of mice

(A) Response profiles of small intestinal afferent nerves that were stimulated with SSA-JE2 (left) or SSA-Psm$^-$ (right) for 90 min. Colors reflect the different origin and cleanliness of the mice that were used in the experiments. Animals that were kept in the SPF facility of the University of Sheffield (colored traces) and animals from the SOPF facility of SAHMRI (dark) responded similarly to both SSA (see methods for more details on the differences between mice). Nerve activity during long term application of SSA-Psm$^-$ was characterized by a pronounced variability in both cohorts of animals.

(B) The excitatory (left) and inhibitory (right) effect of SSA-JE2 and SSA-Psm$^-$ was not significantly different between animals from the two different facilities. Long-term application of SSA-Psm$^-$ inhibited nerve activity of some animals from either

facility which is reflected as large variability of the data. The different shapes represent that experiments were performed on different cohorts of mice in Sheffield (circles and squares) and Australia (triangles).

Fig S4

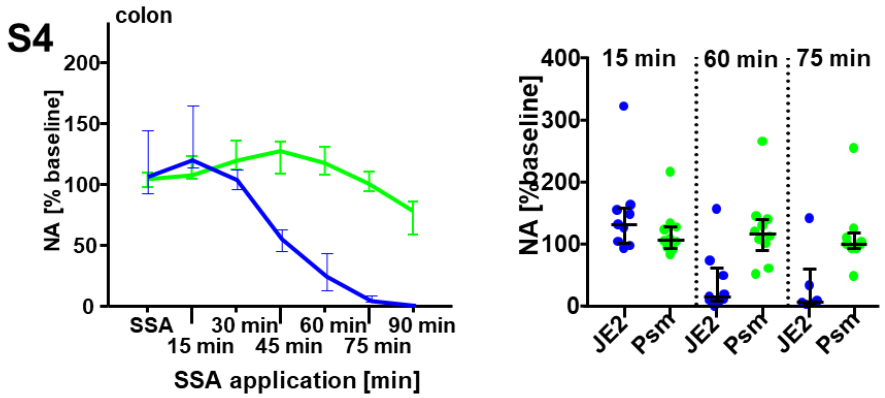


Figure 10(S4): Response of nerves innervating the proximal colon to SSA

(A) Response profile of colonic afferent nerves to SSA-JE2 (blue) and SSA-Psm$^-$(green). Data for SSA-JE2 was obtained from animals in Sheffield and replicated from our previous study. Experiments with SSA-Psm$^-$ were performed in Australia with mice from an SOPF facility. As in the small intestine, the response profiles induced by SSA-JE2 and SSA-Psm$^-$ were different. Nerve activity was normalised to baseline before SSA application.

(B) Comparison of the early (left part of the panel) and late (right part of the panel) effect of SSAs on colonic afferents. There was no statistical difference with regard to the excitatory effect of SSA-JE2 and SSA-Psm$^-$. The SSA-induced changes of nerve activity during long term application were significantly different although the response to SSA-Psm$^-$ was also variable.

Non-parametric statistical analysis (Kruskall-Wallis Test with Dunn's Post-Hoc analysis or Mann-Whitney test)

Fig S5

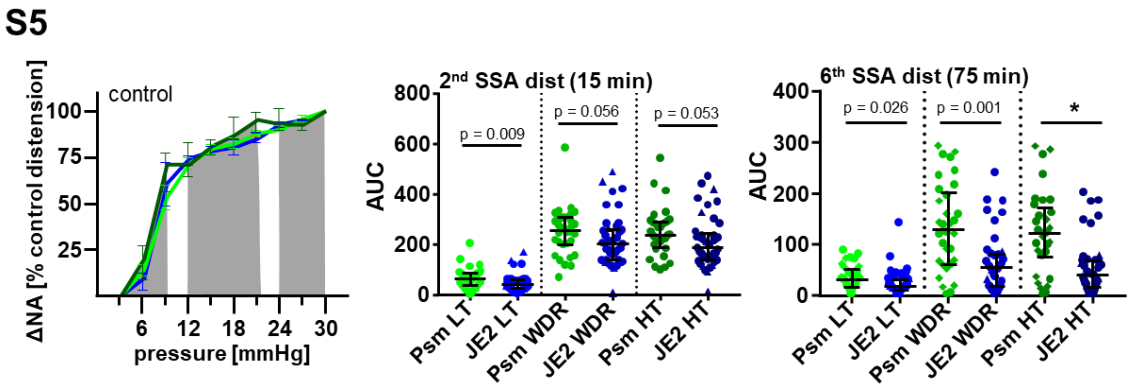


Figure 11(S5): Psms contribute to the inhibition of mechanosensitivity induced by SSA-JE2

(A) The distension response profile of small intestinal nerves before application of SSA is characterized by the sequential activation of low threshold, wide dynamic range and high threshold afferent fibers. The effect of SSA on these aspects of mechanosensitivity were investigated by integrating the pressure-induced increase of afferent discharge as depicted.

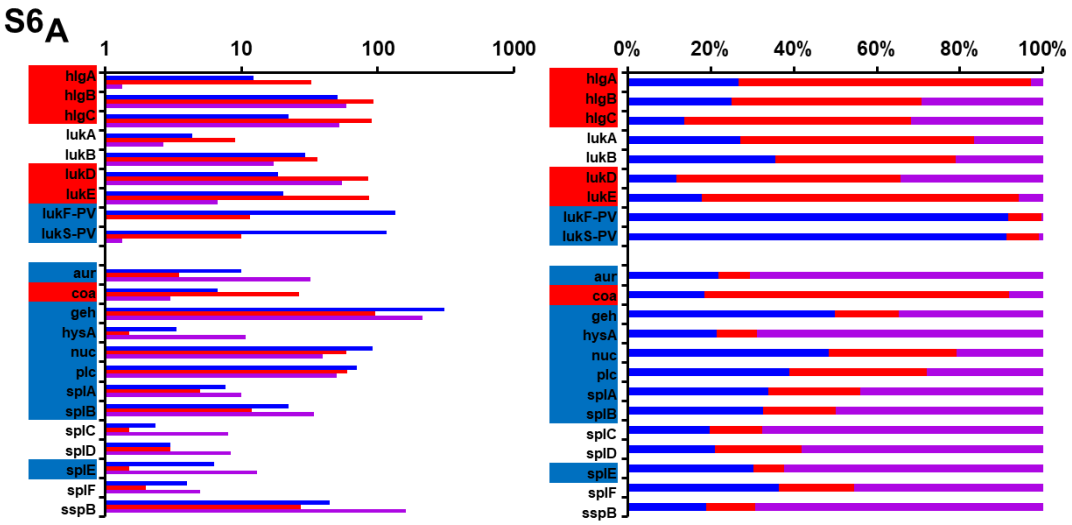
(B) Comparison of distension-induced firing 15 min after SSA-Psm$^-$ or SSA-JE2 application. Only the mechanosensitivity of the LT component was differently affected by SSA-Psm$^-$ and SSA-JE2. The higher activity indicates a reduction of the SSA-JE2 induced inhibition of mechanosensitivity. A similar trend was observed for WDR and HT components but the SSA-induced

alteration of mechanosensitivity was not significantly different between SSA lacking Psm and wildtype suggesting that Psms in SSA-JE2 have a bigger impact on low threshold fibers during short term application.

(C) Distension-induced firing of LT, WDR and HT components was significantly higher when SSA lacking Psms were applied for 75 min which means that the extent of SSA-JE2-induced inhibition of mechanosensitivity is dependent on the abundance of Psms. In a small number of experiments with SSA-Psm$^-$, the distension response of WDR and HT component was reduced to a similar degree as in experiments with SSA-JE2.

Data from experiments where SSA-Psm$^-$ inhibited spontaneous firing are indicated as squares. The distension response of JE2 represents SSA-JE2 (circles) and SSA from the wildtype used for the generation of the Psm-deficient JE2 (triangles). There was no significant difference between these two variants of JE2.

Fig S6



B

Newman	USA300	MW (kDa)	function
☑	☑	36.0	Alpha-hemolysin (hla)
	☑	44.6	Cysteine protease (SspB)
☑	☑	3.0	Delta-hemolysin (hld)
☑	☑	32.6	Gamma-hemolysin component C (hlgC)
☑	☑	29.0	Glutamyl endopeptidase (V8 proteinase)
☑	☑	4.5	Phenol soluble modulin beta 2 (psmβ2)
☑	☑	2.3	Phenol-soluble modulin alpha 2 peptide (psmα2)
	☑	2.6	Phenol-soluble modulin alpha 3 peptide (psmα3)
	☑	2.2	Phenol-soluble modulin alpha 4 peptide (psmα4)
	☑	4.5	Phenol-soluble modulin beta 1 (psmβ1)
	☑	21.9	Serine protease (splF)
	☑	44.3	Staphylococcal lipase 1 (geh)
	☑	55.0	Zinc metalloproteinase (aur)

Figure 12(S6): Exoproteomes of *Staphylococcus aureus* strains USA300 (JE2), Newman and NCTC8325 comprise distinct arrays of secreted proteins. The quantity of individual mediators is strain-specific.

(A) Data from Chapman et al. 2017 for indicating the absolute abundance (log) or relative proportion (%) of cytolysins and exoenzymes in supernatants produced in TSB. Colours indicate increased abundance of this protein in either USA300 (blue) or Newman (red). NCTC8325 is depicted it is that strain that was used to generate SH1000. In contrast to NCTC8325, SH1000 produces smaller amounts of exoproteins. This study did not include Hla or Psms in this analysis.

(B) Mass-spectrometry data from Tsompanidou et al 2013 that investigated the effect of Psms on colony spreading using the same three strains that were used in the present study and their respective Psm mutants. As knock down of Psms reduces colony spreading in USA300, Newman and SH1000, it can be concluded that all strains produce Psms which is not reflected in the MS data. Additionally, there are some marked differences between this data and the Chapman data with regard to the geh lipase and the aur protease highlighting the impact of the method used to assess exoproteome composition

Fig S7

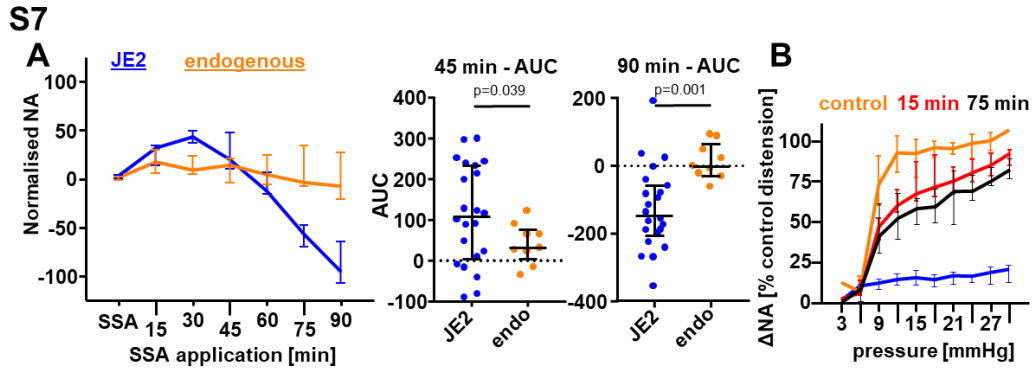


Figure 13(S7): Effect of commensal Staphylococci on afferent nerve activity.

Staphylococci isolated from the intestines of mice from our study using Baird Parker plates. SSAs produced from these cultures had a lower optical density than SSA-JE2 before centrifugation (OD_{600} = 4-5)

(A) Application of these SSA had little effect on spontaneous nerve activity. (B) The distension-induced increase of NA was not changed during prolonged application and was different from the strong reduction of mechanosensitivity during SSA-JE2 application (75 min distension response reprinted for reference.)

N(JE2) = 22, N(endogenous) = 9

Fig S8

A

	size					latency				
	n	0.25	median	0.75	p-value	n	25%	median	75%	p-value
NG_traced	28	1021	1232	1457	0.1784	28	246.3	380	463.8	0.607
NG_untraced	27	940	1078	1299		27	240	320	675	
TL_traced	52	1088	1459	1814	0.0039	52	217.5	355	568.8	0.003
TL_untraced	152	879	1120	1491		152	335	500	793.8	
LS_traced	18	1233	1357	1734	0.0002	18	177.5	237.1	326.1	0.044
LS_untraced	123	645	858	1182		123	195	270	460	
traced all	98	1091	1359	1776	<0.0001	98	224.3	327.2	465	0.040
untraced all	302	791	1021	1390		302	245	360	650	
NG_all	57	936.5	1159	1421	a	57	242.5	345	675	a
TL_all	213	888	1204	1606	a	213	312.5	465	777.5	a
LS_all	141	712	908	1343	b	141	194.7	259.3	409.7	b

B

	size_responding				non-responding					size_all				% responding
	n	0.25	median	0.75	n	0.25	median	0.75	p-value	n	0.25	median	0.75	
NG_traced	28	1021	1232	1457	3	653	810	1026	0.0273	31	933	1161	1449	90.32
TL_traced	52	1088	1459	1814	7	716	1057	1698	0.2087	59	1041	1418	1814	88.14
LS_traced	18	1233	1357	1734	9	756	1491	1872	0.5165	27	1215	1359	1818	66.67
NG_untraced	27	940	1078	1299	11	716	916	1563	0.5329	38	879.8	1068	1363	71.05
TL_untraced	152	879	1120	1491	51	695	879	1150	0.0003	203	832	1057	1425	74.88
LS_untraced	123	645	858	1182	48	451.5	619.5	833	<0.0001	171	561	821	1091	71.93
traced all	98	1091	1359	1776	19	749	1057	1698	0.5478	117	1026	1357	1774	83.76
untraced all	302	791	1021	1390	110	561	791	1064	<0.0001	427	716	940	1299	70.73
NG_all	57	936.5	1159	1421	14	700.3	901.5	1390	0.0735	71	887	1122	1392	80.28
TL_all	213	888	1204	1606	58	695	897.5	1164	<0.0001	271	850	1120	1568	78.60
LS_all	141	712	908	1343	57	486.5	683	935	0.0002	198	607	837	1265	71.21
all	400	837	1093	1493	129	572	810	1149	<0.0001					

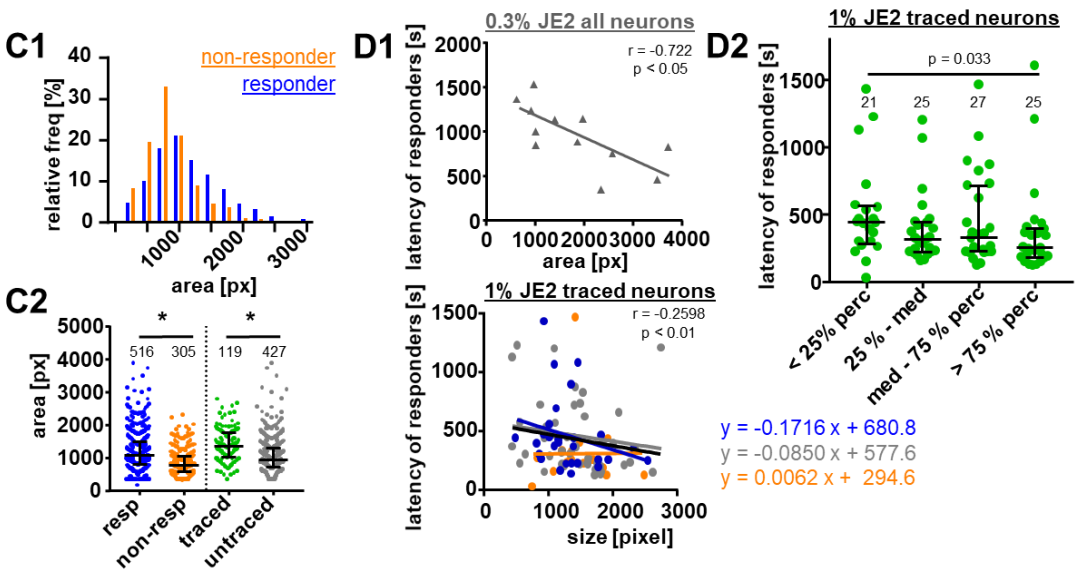


Figure 14(S8): Effect of soluble mediators from SSA on cell membrane permeability

(A) Table comparing sizes and response latencies between different subtypes of responding. Differences in sizes are also reflects in response latencies.

(B) Table comparing sizes of responding and non-responding DRG neurons. Responding cells tend to be larger.

(C1) The size histogram and (C2) average cells size of responding and non-responding cells were different. Traced neurons that responded to SSA were also larger than non responding neurons.

(D1) The Spearman correlation coefficient indicates a significant inverse relationship of response latency and cell size for neurons responding to 0.3 % JE2 (top) and traced neurons responding to 1 % SSA-JE2 (bottom). Blue, gray and orange data points and regression lines represent neurons from NG, TL and LS DRG respectively. (D2) Traced neurons were binned according to their cell size (25% percentile, median and 75 % percentile of traced neurons) and corresponding response latencies were calculated. The response latency of the smallest cells was significantly higher than the largest cells indicating a higher sensitivity of the large neurons to SSA-JE2.

Non-parametric statistical analysis (Kruskall-Wallis Test with Dunn's Post-Hoc analysis or Mann-Whitney test); n numbers as indicated (coverslips or cells) obtained from 4-5 animals

Fig S9

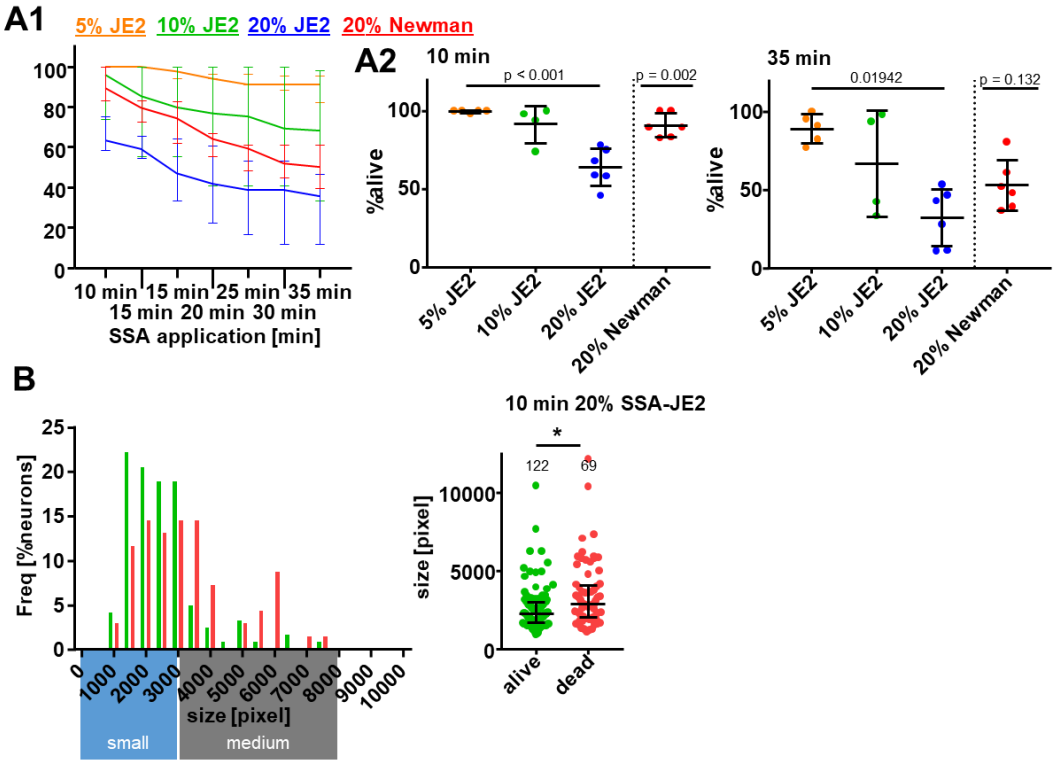


Figure 15(S9): Effect of SSA on neuronal viability assessed by manual analysis of PI fluorescence.

(A1) Percentages of PI-negative cells (alive) decreased as a result of SSA application. (A2) There was a significant effect of concentration on SSA-JE2-induced cytotoxicity at 10 and 35 min. SSA-Newman had a smaller cytotoxic effect at 10 min compared to JE2 (left) but they also reduced the number of viable cells substantially at 35 min (right) where the difference between these strains was not significant.

(B) Analysis of cell size from neurons incubated with 20 % SSA-JE2 for 10 min. Size distribution histogram (left) and median of average cell size (right) show that dead cells were larger than cells that had not taken up PI as an indicator for cell death.

Data points represent percentages for individual coverslips (A) or individual cells (B), N = 2-3 per condition

CHAPTER V

General discussion and future directions

1 Bacterial substances that modulate visceral sensitivity and intestinal function

In this thesis, I have described that soluble mediators produced by pathogenic *S. aureus* at high population density act as modulators of neuronal activity and intestinal function. This was confirmed by using a mutant of SA devoid of quorum sensing-regulated mediators. SSA from the accessory gene regulator (Agr) mutant reversed all phenotypes including

(1) Excitation and inhibition of spontaneous afferent firing
(2) Inhibition of mechanosensitivity
(3) Increase of cell membrane permeability in primary sensory neurons
(4) Increase of transepithelial ion movement
(5) Reduction of contractile activity in mouse colon

Using a microbial approach, I further identified which agr-regulated transcripts contributed to the effects of SSA on visceral sensitivity and intestinal function.

Table 3: Summary of the effects of SA mutants and strains on intestinal afferent activity, intestinal function and in propidium live imaging experiments. haemolysin, Pvl, Agr, Protease and Psm knock-outs were produced on JE2 background.

Strain	Spontaneous discharge		Distension response	Colon contraction	Ussing Chamber	Propidium iodide
	excitation	inhibition				
JE2	☑	☑	↓	down	↑	☑
Newman	☑	☒	↔	Down	↑	☑
SH1000	some	some	↓			
Endo	little	☒	↔			
Hemolysin	Down	☑	↓		↑	☑
Pvl	some	☑	↓			
Agr	☒	☒	↔	No	↔	☒
Protease	☑	variable	↓			
Psm	☑	☒	little ↓		↑	☒
Boiled	☒	☒	little ↓			

1.1 Bacteriological approach to identify neuromodulatory substances

In order to study an interaction between bacterial mediators and neurons, we chose to investigate supernatants of bacterial cultures containing a number of secreted virulence factors which may have synergistic or antagonistic effects (Perret et al., 2012; Vandenesch et al., 2012b). The composition of supernatants produced under identical growth conditions is mostly affected by the genotype and this enabled me to investigate the contribution of individual components to the overall effects of SSA. Other environmental factors however also impact upon the supernatant composition because bacteria express sensory systems to respond to local conditions (Villanueva et al., 2018).

Indeed, we found differences in the neuromodulatory effects between supernatants prepared in two bacterial growth media and different preparations of SSA. Tryptic soy broth (TSB) and brain heart infusion buffer constitute proteolytic digests of brain/heart infusions or soybean that are supplemented with additional nutrients[10]. It is possible that some batches contain trace elements that potentially interfere with *S. aureus*' virulence factor production. In addition, the ultimate nutrient composition will also be affected by the autoclaving process prior to supernatant preparation. Although we did our best to standardise supernatant preparation, these factors may all contribute to the observed variability

[10] www.bd.com, www.oxoid.com

which was a consistent observation in all three studies. Preparation of supernatants in chemically defined media may be an alternative but this could also decrease the amounts of virulence factors that are produced. It would also be interesting to study the effect of metabolic stressors such as bile acids, antibiotics, anaerobic culture etc. on the neuromodulatory effect of SSA. However, because we have identified the major components of SSA that caused excitation and inhibition of afferent nerve activity, it may be possible to predict these effects based on examining the abundance of these components in SSA prepared under those conditions. Overall, this illustrates that in future studies more emphasis on describing conditions of supernatant preparation should be done.

1.2 Bacterial mediators and mechanisms of neuromodulation

The data presented in our studies argues for distinct roles of individual mediators in SSA-induced neuromodulation. SSA-Psm$^-$ induce excitation but not inhibition whereas the opposite was found for Hla-deficient SSA. This also suggests that different mechanisms may contribute to excitation and inhibition because we observed profound differences between these two SSA in the PI experiments (chapter IV). Below we will further discuss potential mechanisms involved in Psm-induced inhibition and Hla-induced excitation.

1.2.1 Phenol soluble modulins

Phenol soluble modulins contributed to nerve inhibition and had an overwhelming effect on cell membrane permeability as assessed by propidium iodide suggesting that disruption of the cell membrane underlies nerve inhibition. We acknowledge that this dye is also frequently used to investigate cell death but our live imaging approach enabled us to detect small changes of fluorescence intensity early following application. In addition, we intended to validate the differences between supernatants from different bacteria and therefore, cell viability is a valid but secondary concern.

In the PI experiments, we found that SSAs lacking Psms, similar to SSA from the quorum sensing mutant, failed to increase PI fluorescence. This is in agreement with their direct regulation by AgrA (Le and Otto, 2015) and strong membrane-disrupting capacity (Verdon et al., 2009). In fact, the gene for one of the Psms, δ-toxin, is embedded into RNAIII and is therefore directly activated by AgrA. RNAIII regulates the expression of other genes, some of which (haemolysins and leucocidins) have also pore forming properties and therefore, the overwhelming effect of Psm knock-out was unexpected and may indicate that the Psm-induced changes are more persistent. It is also documented that the Psm-α

locus regulates the production of Hla and the Psm mutant may therefore produce less Hla but our experiments with the Hla-deficient mutant suggest that α-haemolysin does not contribute to pore formation to a similar degree (Berube et al., 2014). Furthermore, preliminary experiments using Psm-α or Psm-β single mutants indicate that it is the β-peptides that predominantly contribute to pore formation-induced increase in PI fluorescence (data not shown). We attempted to narrow down which group of Psms was involved in SSA's effects on small intestinal nerve activity but in these experiments, none of the single mutants recapitulated the phenotype of the complete mutant suggesting that inhibition of nerve activity results from the combined effect of several peptides. In the majority of this set of experiments however, the Psm-all mutant also continued to inhibit intestinal afferent firing which indicates that host factors do also play a role at determining sensitivity to Psms (chapter IV, Figure 11).

To the best of our knowledge, it is not known exactly how Psms interfere with the membrane. Based on studies using the Psm δ-toxin, the initial increase of cell membrane permeability may not involve the formation of structured pores. Instead, Psms attach and transiently insert into the lipid double layer (Verdon et al., 2009). At high concentrations, Psms act in a detergent-like manner (Biggin and Sansom, 1996) which could account for their large and perhaps more detrimental effects on cell membrane permeability and a sustained modulation of afferent nerve activity. The Psm-induced disruption of the cell membrane may also interfere with the induction of action potentials and cell viability causing the profound decrease of nerve activity.

Psm-deficient supernatants also had a smaller inhibitory effect on mechanosensitivity compared to wildtype SSA (chapter IV, Figure 2) suggesting that the Psm-mediated inhibition is not specific for a distinct subpopulations of visceral afferents. The mechanisms underlying intestinal mechanosensitivity are not completely understood (chapter I, p 41) but it is suggested that the afferent terminal comprises mechanosensitive channels that are activated by changes of the cell shape which are transmitted either through the cytoskeleton or the lipid bilayer (Alcaino et al., 2016; Brierley, 2010). By interfering with membrane tension, Psms are prime candidates for affecting afferent mechanosensitivity.

Psms are not only produced by the USA300 strain of *S. aureus*. Other SA strains but also more distant relatives such as *Staphylococcus epidermidis* and *Staphylococcus lugdunensis* have been shown to produce similar substances. In our study, we used the Psm-producing Newman strain to further investigate the link between modulation of afferent nerve activity and membrane permeability (chapter IV, Figure 4, p 132). Unexpectedly with regard to Psm-production, SSA-Newman did not

inhibit small intestinal afferent firing during prolonged application despite an apparent increase of PI fluorescence. Due to a mutation in an additional transcriptional regulator of toxin-expression, *S. aureus* Newman produces large amounts of Hla and virulence factors that we have not investigated in our candidate approach. We propose that these underlie the lack of inhibition because they prolong excitation or interfere with membrane disruption. Cross reactivity has indeed been described for γ-haemolysin and leukocidinF but may also be possible between other PFT. Therefore, it is possible that such a mechanism may account for the apparent incongruent observation with *S. aureus* Newman. In addition to *S. aureus*, other gram$^+$ bacteria for example commensals and even humans, produce detergent-like substances with antimicrobial activity. Exactly how it would be possible for these substances to distinguish between pathogenic and commensal bacteria is not clear yet.

The onset of inhibition was delayed compared to excitation which could be indicative of an accumulative effect. Alternatively, it is possible that inhibition requires the release of mediators from other intestinal cells such as immune cells. Interestingly, mast cells have been shown to degranulate when stimulated with Psms (Hodille et al., 2016; Nakamura et al., 2013) and mast cell mediators, particularly proteases, have been shown to exert inhibitory effects on DRG neuron excitability via PAR-4 (Sessenwein et al., 2017). Other mast cell mediators however can also excite afferent nerve activity (Song et al., 2015).

Psms have also been shown to activate FPRs and we have found that those are expressed in sensory ganglia with higher susceptibility to SSA suggesting that receptor activation may contribute to neuro-modulation induced by Psms. Excitation was not reduced in the when using SSA-Psm$^-$ and this may argue against an involvement of FPR in SSA induced excitation. FPRs and -like receptors (FPRLs) are G-protein-coupled receptors and thus, induce several signalling pathways dependent on the cellular expression of adapter molecules that could potentially contribute to inhibition of nerve activity. Gi and Go-coupling of FPR induces IP_3 production and activation of PI3K. In addition, FPR can also activate the MAPK cascade and small guanosine triphosphate (GTP)ases which are important for the regulation of cell proliferation, differentiation as well as apoptosis and trafficking respectively (Migeotte et al., 2006). However, little is known about FPR signalling in neurons and it remains to be determined whether and how FPRs can contribute to inhibition of nerve activity. Importantly however, as it is the case for other G protein-coupled receptors, FPR can be activated by several ligands and those induce unique intracellular signalling events (Cattaneo et al., 2013). Additionally, FPRs display a high constitutive activity possibly caused by the ubiquitous presence of ligands. This lack of specificity and

desensitisation of FPRs upon activation may account for the limited contribution of Psm to excitation in contrast to inhibition (Wenzel-Seifert et al., 1998). In contrast, internalisation and therefore cessation of the constitutive excitatory effect may contribute to the decrease of nerve activity. To study the potential involvement of FPRs in SSA induced neuromodulation, it would be of interest to apply synthetic ligands of FPRs to intestinal nerve preparations. Binding of Psm to FPR is not necessary for their membrane-disrupting activities (Otto, 2014).

1.2.2 α-haemolysin

The pore-forming toxin Hla contributed to SSA-induced excitation which is consistent with its excitatory activity in cell-based experiments with dorsal root ganglia neurons (Chiu et al., 2013). The inhibitory effect of SSA on afferent nerve activity as well as the increase of intracellular PI were not particularly affected by the lack of Hla (chapter IV). This is intriguing because Hla as well as Psms both interfere with the cell membrane. What does this mean with regard to the mechanism of excitation and inhibition? It is likely that the Hla- but not the Psm-induced changes of membrane permeability specifically enable the entry of positively charge ions leading to depolarisation and excitation of the nerve. Indeed, it is known that pores formed by PFT such as Hla are selective for molecules with a specific diameter and charge (Menestrina et al., 2003). The channels formed by polymerisation of Hla monomers for example allow the passage of ions which can directly contribute to depolarisation of the cell membrane, and nucleotides such as ATP. Interestingly, it has also been found that Hla-pores can be inhibited by high concentrations of calcium and pore formation is dependent on membrane potential (Menestrina, 1986). This may account for the smaller contribution of Hla to the increase of intracellular PI compared to Psms and could prevent a sustained excitation which is known to desensitise afferent nerves (Sapio et al., 2018; Spencer et al., 2018).

Haemolysin-induced pore formation requires the expression but not activity of A disintegrin and metalloprotease-10 (Ezekwe et al., 2016). We found that ADAM10 is expressed at high levels in DRG, retrogradely labelled neurons and intestinal tissue which is constituent with ADAM10 regulating tissue integrity, cell survival and inflammation in a number of tissues (Saftig and Lichtenthaler, 2015). The proteolytic activity of ADAM10 induces cleavage of the tight junction protein E-Cadherin (Inoshima et al., 2011), activation of acid sphingomyelinase (Becker et al., 2018) and of the inflammasome NLRP3 (Ezekwe et al., 2016). The subsequent loss of tissue integrity, ceramide-induced cell death and

inflammation constitute signals that can activate sensory nerves and thus, Hla-induced activation of ADAM10 could contribute to SSA-mediated excitation.
In the nervous system, ADAM10 has pivotal roles in neuro- and gliogenesis. It cleaves Notch which is directly involved in the differentiation process and also the amyloid precursor protein APP to a soluble intoxic variant which contributes to neuronal survival (Yuan et al., 2017). It may be hypothesised that neurons therefore have developed mechanisms to prevent modulation of ADAM10 by Hla.

In addition to ADAM10, it has also been found that sphingomyelinsynthesase 1 (Sgms1) is important for Hla-induced fore formation. Knockdown of Sgms1 significantly reduced Hla toxicity in cell based assays (Virreira Winter et al., 2016). Sgms 1 substantially contributes to the formation of sphingomyelin which is an essential component of structural membrane domains called lipid rafts. Indeed, Hla has been shown to preferentially bind these liquid-ordered membrane compartments and thus regulating their abundance may constitute a mechanism to increase or decrease the susceptibility of a cell to Hla. This will be further discussed in section 1.4.2 (p 172) of this chapter.

1.2.3 proteases

To our surprise, we found that the presence or absence of bacterial proteases did not significantly impact on the excitatory or inhibitory effect of SSA on afferent nerve activity (chapter IV, p 125). Previous studies by other groups have shown that host serine proteases such as thrombin and trypsin activate protease-activated receptors (PAR) by proteolytic cleavage of their N-terminal domain. PARs (PAR_{1-4}) are expressed by sensory and enteric neurons and application of proteases or PAR-activating peptides such as SLIGRL can induce neuronal activation particularly via PAR_1 and PAR_2 (Buhner et al., 2018; Cenac, 2013; Rudack et al., 2007). This has also been demonstrated for some bacterial proteases such as gingipain from *Porphyromonas gingivalis* and serralysin from *Serratia marcescens*. However, other bacterial proteases have been shown to inhibit PAR-signalling by alternative cleaving of the receptor (Shpacovitch et al., 2007). In addition, activation of PAR_4 by host and bacterial proteases has been shown to reduce neuronal excitability in-vitro and in-vivo inhibit neuronal activity (Asfaha et al., 2007; Sessenwein et al., 2017). To our knowledge, a possible interaction between staphylococcal proteases and PARs has not previously been studied. The main proteases produced by the USA300 strain of *S. aureus* constitute metallo- (aureolysin), serine (V8, SspA and SspB) and cysteine (staphopains) proteases which have been shown to cleave a number of immune proteins including complement factors, prothrombin, chemotaxis-mediating proteins and antibodies (Kolar et al., 2013;

Pietrocola et al., 2017) suggesting that they may also be able to cleave PARs. However, it is possible that both, excitatory and inhibitory, PARs are activated by staphylococcal proteases in wildtype SSA or that they inactivate PARs and therefore, do not contribute to SSA-induced neuromodulation. It could be that SSA from single mutants are able to unravel whether staphylococcal proteases modulate PARs because they would lack either activating or inactivating proteolytic activity. Alternatively, staphylococcal proteases do not contribute to SSA-induced neuromodulation directly but rather mediate tissue penetration of other virulence factors. This could explain why we observed a particularly high variability of the inhibitory response when SSA from the protease-deficient mutant were applied (chapter IV, Figure 2, p 121).

1.2.4 Other components of SSA

The strong dependence of nerve inhibition and pore formation on Psms was a little unanticipated given our finding that heat treatment also abolished the inhibitory effect of SSA. Psms and the α-peptides in particular constitute short peptides (22-40 aa) that would be expected to be rather resistant to heat-treatment. It is therefore possible that it is the longer heat-sensitive Psms of the β-group that are more important for nerve inhibition. However, SSA continued to inhibit spontaneous discharge in some animals even in the absence of Psms suggesting that other, heat-labile components, can also inhibit nerve activity. Other heat-labile and -resistant mediators may also contribute to the inhibition of mechanosensitivity because we observed some degree of reduction of distention-induced firing even in experiments with boiled or Psm-deficient SSA (chapter IV, Figure 1, p 117).

Heat-stabile

Our observations suggested that heat-stabile mediators in SSA also contributed to the alleviation of mechanosensitivity. SSA contain two classes of heat-stable mediators which will be described below.

Firstly, *S. aureus* is known to produce a number of heat-resistant enterotoxins (SE) that have been shown to induce emesis (Hu et al., 2014). The mechanisms underlying this have been largely investigated in house musk shrew (because mice are not vomit-competent) and suggest a role of vagal afferents and intestinal 5-HT in the emetic response. Intestinal serotonin plays important roles in regulating enteric reflexes and also activates intestinal sensory nerves via 5-HT receptors (Heredia et al., 2013; Hicks et al., 2002; Keating et al., 2008). These constitute a family of GPCRs -protein-coupled receptors which activate intracellular signalling pathways that could potentially lead to inhibition of mechanosensitivity and the ligand-gated ion channel 5-HT_3R. Interestingly, activation of the 5-HT_3

receptor, has recently been shown to reduce distension-induced firing of bladder afferents (Konthapakdee 2017, unpublished) suggesting that 5-HT release by staphylococcal enterotoxins may account for the decrease of mechanosensitivity in experiments with boiled SSA.
We did not particularly focus on SE because the USA300 (JE2) strain does only produce two (SEK and SEQ) out of the 20 described SE. Interestingly, the Newman strain produces SEA which, in the house musk shrew, is involved in the serotonin-mediated emesis which indicates that the prolonged excitation during SSA-Newman application may be the consequence of serotonergic signalling. Other strains such as MW2 and N315 are able to produce a larger array of these substances (Chapman et al., 2017, www.kegg.jp).

Secondly, cell wall components produced by *S. aureus* such as peptidoglycans are also resistant to heat treatment and play an important role in host-bacteria interactions. Peptidoglycan structures of commensal and pathogenic bacteria are different and activate extracellular (TLR2) and intracellular (NOD1, NOD2) receptors (Baik et al., 2015; Kielian et al., 2005; Schwandner et al., 1999). It is not known yet whether either of these signalling pathways affect mechanosensitivity directly. Evidence from studies with the cell wall components of gram$^-$ bacteria (LPS) suggests that TLR4 signalling might be involved in alteration of visceral sensitivity but other receptors (TRPA1) may also be involved (Liu et al., 2005; Meseguer et al., 2014; Ochoa-Cortes et al., 2010). In contrast, little is known about NOD signalling in neurons and it is also a matter of debate how PGNs penetrate the cell membrane. It is suggested that toxin-induced pores, ion channels, peptide transporters or delivery via outer membrane vesicle play a role (Davis and Weiser, 2011). Intracellular PGNs have been shown to bind proteins of the cytoskeleton particularly myosin, tubulin and vimentin which play important roles in mechanosensitivity (Baik et al., 2015). In addition to direct effect of PGNs on neurons, they may also affect mechanosensitivity through the release of mediators from resident immune cells. In this regard, the activation of mast cells by staphylococcal PGNs is an interesting observation (Wu et al., 2007). We have not focussed on PGNs and other cell wall components because their expression is inhibited at high population density via AgrA. However, SSA will contain PGN because they are produced at earlier time points of bacterial growth as a result of shedding processes. Future studies may further investigate the role of staphylococcal cell wall components on afferent nerve activity using isolated PGNs.

Heat-labile

We suggest that additional heat-labile components in addition to Psms contribute to inhibition of afferent nerve activity because Psm-deficient supernatants but not boiled SSA inhibited spontaneous nerve activity in some animals (chapter IV, Figure 1 and Figure 2).

It is possible that in the absence of Psms and in sensitive hosts, other pore-forming haemolysins and leucocidins can also contribute to nerve inhibition. However, these form pores with a similar architecture as Hla (Vandenesch et al., 2012b) and are therefore more likely to contribute to excitation rather than inhibition. Additionally, SSA-Newman which produces large amounts of those PFT (Chapman et al., 2017b) did not inhibit nerve activity and this argues against their involvement in nerve inhibition.

Other bacteria such as *Clostrium* species produce neurotoxins that interfere with sensory neuro-transmission (Chiu et al., 2012) and in fact, some pharmacologic receptor and channel inhibitors originate from bacteria or other microorganisms suggesting that related substances may also be produced by *S. aureus*. To our knowledge, *S. aureus* proteins have not been investigated for a similar activity.

Additionally, the quorum sensing molecules involved in the activation of the AgrA two-component system, auto-inducing peptides (AIP), may potentially also contribute to nerve inhibition. It is currently unknown whether there are receptors for AIP from gram$^+$ bacteria on the host cell membrane. In contrast, the quorum sensing molecules of gram$^-$ bacteria (N-acylhomoserine lactone, AHL) have been shown to bind and activate the bitter taste receptors hT2R38 and mTas2r138 which are not only expressed in taste receptor cells but also in nasal chemosensory cells, mouse intestine and phagocytic neutrophils suggesting that bacterial sensing is important in these organs (Gaida et al., 2016; Prandi et al., 2013; Tizzano et al., 2010). While in taste receptor cells activation of Tas ultimately serves taste perception, it possible that the ability to "taste" bacterial density initiates host defence mechanisms in other tissues.

Ultimately, bacteria have been shown to produce neurotransmitter such as serotonin, GABA, dopamine and noradrenaline (Cawthon and de La Serre, 2018b; Pokusaeva et al., 2017). Receptors for those molecules are expressed on intestinal afferent nerves and thus, this may constitute another mechanism underlying Psm-independent nerve inhibition. These compounds are relatively short-lived and thus it may be that they were inactivated in animals were no inhibition was observed. For a further discussion of host factors that may contribute to SSA-susceptibility refer to section 1.4.3 (p 175).

To conclude, SSA contain a number of compounds, in addition to those studied in our experiments, that could potentially modulate neuronal activity. Future studies may use broad spectrum antagonists to investigate a role of bacteria-produced neurotransmitters and taste receptors. Alternatively, we propose that a microbial approach could be used to study the involvement of lipid mediators and enterotoxins.

1.3 Neuromodulation during intraluminal application

In our study, we found remarkable differences between intraluminal and bath application of SSA (chapter II, p 71). A biphasic response to 10 and 20 % SSA characterised by a strong excitation and inhibition, was observed during bath application of SSA whereas during intraluminal application of 100 % SSA only a smaller excitatory response was evident. Our initial thoughts were that the smaller degree of excitation may be indicative of different mechanisms of action. While during bath application of SSA a direct interaction between afferent nerves and mediators was anticipated; intraluminal perfusion may induce the excitation through the release of gastrointestinal hormones from enteroendocrine cells in the epithelium which are known to activate sensory nerve activity. The renewal of the inhibitory component after disruption of the epithelial barrier in experiments where SSA were applied at a later time point, supported this hypothesis. Additionally, Yano et al. (2015) found that metabolites from segmented filamentous bacteria were able to induce serotonin release from serotonin-releasing enterochromaffin cells. (Yano et al., 2015).

Some of our data however do not support an involvement of this indirect mechanism. The high concentration (100 % SSA) that was needed in order for SSA to affect afferent nerve activity during intraluminal application argues against a specific interaction of these compounds with receptors expressed on the apical surface of EEC. Although we have not assessed for the absolute quantities of neuromodulatory components in SSA ourselves, the fact that 20 % SSA exert strong effects during bath application and effectively killed primary DRG neurons in-vitro indicates that those mediators do not induce EEC transmitter release either through receptor-mediated or -independent (lytic) activities. Additionally, we found that apical application of SSA to mucosa-submucosa preparations in Ussing Chambers did not affect short circuit current compared to vehicle which is in line with earlier studies using the T84, PC12 and Caco-2 cell line (Ahnert-Hilger et al., 1985; Bell and Quinton, 1992; Kwak et al., 2012). If they induced hormone release from the epithelium, an increase of SCC would be expected because those hormones can modulate secretion through receptors expressed on the intestinal

epithelium or on enteric neurons that induce secretory responses (Baldassano et al., 2012; Lundgren et al., 2000; Schulzke et al., 2014; Thiagarajah et al., 2015). This also indicates that EEC are resistant to Psm-induced cell lysis (Bader et al., 1986) and that Hla-induced activation of the ADAM10 protease or bacterial proteases does not reduce epithelial barrier function.

These findings suggested that it is likely the passage of mediators from the supernatants across the epithelial barrier that contributes to neuromodulation during intraluminal application. This hypothesis is further supported by the observation that during intraluminal application mechanosensitivity of low threshold fibers but not high threshold fibers is reduced. These fibers are thought to constitute mucosal or muscular afferents and respond to stimuli with lower intensity (Brierley et al., 2004). In contrast, both populations of mechanosensitive afferents were affected in experiments were the epithelial barrier was disrupted which allows the diffusion of mediators in deeper layers of the intestinal wall that is innervated by high-threshold serosal and mesenteric afferents (Brierley et al., 2004; Rong et al., 2004). We have not investigated which mediators are involved in neuromodulation during intraluminal application but Hla and Psms do probably play a role because the nerve response pattern in experiments with disrupted epithelium was similar to bath application. The smaller excitatory in these experiment may be indicative of overwhelming inhibitory effect of Psms on mucosal afferents.

1.4 Mechanisms of selectivity

In our studies, we used ex-vivo preparations from mice (and guinea pig) to assess the effects of SSA on intestinal function and sensory signalling and observed a high level of variability of the responses particularly with regard to modulation of neuronal activity. This could indicate that bacterial components selectively interact with particular subsets of neurons or distinct cell types and that host-inherent factors contribute to the response patterns.

1.4.1 Do bacterial components interact with distinct subsets of sensory neurons?

To investigate whether particular subsets of sensory neurons were more susceptible to SSA than others, we performed propidium live imaging experiments on primary neurons from different anatomical locations, with different peripheral projections and cell size. Quantitative RT-PCR was performed on the same subsets of neurons.

Neuronal cell-size

We found significant differences between cell sizes of neuronal populations that responded to SSA and those that did not. Responders and populations with short response latency also tended to be larger than resistant neurons (chapter IV, Figure 4, p 132) indicating that it is the larger cells that are more susceptible to SSA. In whole DRG, large neurons constitute somatosensory neurons that have myelinated processes. Visceral neurons are generally of small to medium size and have unmyelinated or lightly myelinated fibers (C-, Aδ-fibers) which may suggest that these cells are less susceptible. However, in our analysis, neurons labelled by ip injection of CTB (visceral neurons) were more susceptible and surprisingly, they were larger than untraced neurons. Because the size differences were also rather small compared to published ranges, this suggests that we were not comparing "large" somatosensory and "small" visceral neurons but rather different subpopulations within the small-to-medium sized neurons. We did not deliberately focus on these when selecting cells for analysis but it may be that culture techniques or tracing introduced a bias towards cells of this size range. Indeed, others that have used retrograde labelling techniques report an average cell size of colon-innervating lumbosacral neurons of 846±352 μm^2 (approximately 32.8 μm diameter) which would not classify as small neurons (Hibberd et al., 2016; Tan et al., 2008). In the study by Hibberd et al. (2016), colonic DRG neurons with different sizes contributed to different aspects of mechanosensitivity. The smallest neurons constituted a population of $CGRP^-$ afferents that fired with high frequency in response to distension. Thus our finding that the high component of the distension response was not affected by intraluminal application is consistent with small cells being less susceptible than larger cells. In the Hibberd study, the largest cells expressed $GGRP^+$ and did not respond to distension and were hypothesised to be "silent nociceptors", a class of neurons that can be recruited and add to pain sensation under inflammatory conditions. Based on our results, we may hypothesise that it is these $CGRP^+$ neurons that are most sensitive to SSA. CGRP is frequently co-expressed with the transient receptor potential channel TRPV1. The TRPV1 ion channel is highly expressed in visceral afferents but its importance for visceral pain is still a matter of debate (Brierley et al., 2010; Christianson et al., 2006b). Knock-out of TRPV1 reduces whole nerve distension-induced firing in the small intestine and bladder as well as EMG responses to colorectal distension but does not affect the response of colonic afferents to mucosal stroking or the response of high threshold fibers in the small intestine and bladder (Daly et al., 2007; Jones et al., 2005; Rong et al., 2004). In-vivo, TRPV1 knock mice have an increased risk of dying from infection and display cardiac dysfunction after treatment with low dose LPS (Chen et al., 2018; Guptill et al., 2011). On the contrary, they also display less *S. aureus*-induced spontaneous pain and longer survival in a model of lethal *S. aureus* pneumonia (Baral et al., 2018; Chiu et al., 2013).

Thus, TRPV1 and CGRP are important for the regulation of inflammation and pain but further studies are needed to establish whether they have beneficial or detrimental effects. Our finding that *S. aureus* may preferentially target those neurons is therefore of relevance for translational approaches.

Receptor-expression

Selectivity of *S. aureus* for defined cell types has been linked to the expression of receptors for individual virulence factors (Spaan et al., 2017). We found that dorsal root ganglia expressed ADAM10 and formyl peptide receptors which bind the pore forming toxins α-haemolysin and phenol soluble modulins respectively. The expression of ADAM10 was significantly higher than FPR expression and we also detected ADAM10 in lysates of 20 intestine-projecting neurons. The lack of Hla only marginally reduced the ability of SSA to increase intracellular propidium iodide fluorescence. This suggests that ADAM10 expression is not a limiting factor which is in line with its role in neurogenesis (Yuan et al., 2017). Instead, Psms were primarily mediating SSA's activity on cell membrane permeability. Psms have been shown to activate FPRs which we found were expressed at higher levels in DRG than other tissues. However, we were not able to detect FPR expression in lysates of 20 neurons from primary cultures of dorsal root and nodose ganglia and this is consistent with the findings of a recently published single cell RNASeq study (Hockley et al., 2018). They find that the expression level of FPRs is low in single neurons and virtually absent in populations that express little TRPV1 and heparanase. In agreement with other studies, this suggests that the membrane effects of Psms are not mediated through FPR but either receptor-independent or through other receptors. Indeed, the membrane composition has been shown to affect Psm-induced cytotoxicity (see below) and based on the structure of Psms it might be possible that they interact with mas-related G protein-coupled receptor (MRGPR). Human MRGPRX2 has been shown to be activated by amphipathic helical peptides with positive charge such as cathelicidins and human defence proteins which are produced by immune cells and Paneth cells in the intestinal epithelium (Bader et al., 2014; Solinski et al., 2014; Subramanian et al., 2013). Interestingly, LL-37 has been used to activate both MRGPRs and FPRs (Migeotte et al., 2006; Subramanian et al., 2011). Interestingly, a similar crosstalk of host and bacterial poreforming toxins has also been suggested to modulate the activity of ADAM10 (Reiss and Bhakdi, 2012).

Lipid composition

Receptor-independent specificity of Staphylococcus aureus virulence factors towards distinct cell types is linked to cell membrane composition (Laabei et al., 2014; Nishiyama et al., 2012; Vandenesch et al., 2012b). Our data suggests that the membrane of neurons in culture is more susceptible to Psms than

to Hla. In addition, we found that HEK293 cells were more sensitive to SSA than primary neurons. The membrane-permeabilising capacity of Psms is dependent on disordered membrane compartments that are predominantly composed of phosphatidylcholine-glycerolipids. Vesicles or cells containing high amounts of cholesterol and sphingolipids (sphingomyelin) i.e. lipid ordered compartments, are less susceptible to Psm-induced lysis (Almeida*, 2005; Duong et al., 2012; Laabei et al., 2014). Sphingomyelin synthesis is catalysed by sphingomyelin synthesase (Sgms-1, SMS-1) which we found to be expressed at higher levels in neurons than intestinal tissue. In our analysis, Sgms1 expression was not elevated in visceral neurons compared to somatosensory neuron although the former were more susceptible to Psm suggesting that Sgms-1 expression is not a major contributor to the different susceptibility. However, the data from the Hockley study[11] demonstrates that Sgms-1 expression varies between subpopulations of colonic neurons and is the highest in neurofilament heavy chain (Nefh)-expressing neurons which express lower levels of CGRP (Calca) and TRPV1 compared to Nefh-negative "peptidergic" neurons. This indicates that it is this population of visceral neurons that is less sensitive to Psm.

We were also interested to investigate Sgms-1 expression because it is has been shown to be involved in Hla-mediated pore formation (Virreira Winter et al., 2016). Cells devoid of Sgms-1 are more resistant to Hla because of a reduced membrane localisation of ADAM10. We hypothesised that different expression levels might affect susceptibility to Hla but as Hla was not primarily involved in SSA-induced increase of PI fluorescence and Sgms-1 expression was similar between all neuronal populations, we cannot conclude whether this is the case. This might also account for the importance of Sgms-1 for lipid raft formation and ceramide detoxification (Ding et al., 2008; Kidani et al., 2012; Olsen and Færgeman, 2017).

Conclusion

Our data are indicative of a somewhat specific interaction between bacterial modulators and neurons with distinct properties (cell size, receptor expression and membrane composition) but further experiments are required to confirm a potential selectivity. Although propidium live imaging provided interesting insights, other assays could also be used to assess mediator-induced neuronal activation. For example, Chiu et al. (2013) and others have previously used calcium imaging to study neuronal activation by Hla, Psms and leukocidins (Blake et al., 2018; Chiu et al., 2013). They found that these PFTs preferentially target capsaicin-sensitive neurons. In our preliminary experiments however, the calcium indicator dye leached out of the cell and therefore, it might be an alternative to use gene-

[11] https://hockley.shinyapps.io/ColonicRNAseq/

encoded calcium sensing proteins (GCaMP or EPac) which have previously been used to study mechanisms underlying the activation of enteroendocrine L cells (Brighton et al., 2015; Ezcurra et al., 2013; Pais et al., 2016). In addition, electrophysiological recordings of changes of the membrane potential and excitability could be used to better understand the effects of PFTs on neurons and whether this selectively affects distinct subpopulations.

It is also possible to address the question of selectivity in recordings from afferent nerves. Previous studies have used single unit analysis to determine which subtypes of fibers were affected (Brierley et al., 2004, 2005; Daly et al., 2007; Dong et al., 2015; Hillsley et al., 1998). We have not pursued this analysis because the afferent nerves in our preparation displayed a high spontaneous activity which increased even further during distensions causing a considerable amount of signal summation and single units could not be readily discriminated. We also noted that there was a general decrease of the action potential amplitude during prolonged SSA application. Because it is the shape of the action potential that is used to identify individual fibers throughout the course of the experiment, SSA-induced shape changes would interfere with this kind of analysis. To further investigate the effects of SSA on individual fibers, it would be interesting to look at flat-sheet preparations of the colon. They display less spontaneous activity and firing is induced by the application of distinct mechanical stimuli. Furthermore, this preparation can also be used to assess the response pattern of these fibers to receptor agonists such as capsaicin, AITC, serotonin etc. which is characteristic for specific classes of afferents.

1.4.2 Are there differences between cell types?

The factors discussed about for neurons (cell-size, receptor expression and lipid composition of the membrane) are also characteristic for individual cell types and this accounts for their different susceptibility to *S. aureus* (Thänert et al., 2017). For example, it is known that neutrophils and eosinophils are highly susceptible to SSA-induced cell death (Prince et al., 2012) but mast cells and particularly secretory cells appear to be more resistant (Bader et al., 1986; Goldmann et al., 2016). We will discuss our findings with regard to cell types in the intestinal tract

Enteric neurons

We assessed the effect of SSA on enteric neurons indirectly using functional experiments. We found that SSA-induced secretion was not reduced by blockers of neuronal activity or neurotransmission (Ussing Chamber experiments, chapter II, Figure 3, p 73) and that SSA reduced contraction amplitude

but not frequency in motility experiments. These were intriguing findings given the strong neuromodulatory effects of SSA on extrinsic intestinal afferents and suggest that enteric neurons are less susceptible to SSA than sensory neurons. Although it is not clear what accounts for this profound difference, it is consistent with the variability of the responses from sensory neurons. It is possible that the chronic exposure of enteric neurons to bacterial substances has led to the development of defence mechanisms that may also be protective against SSA. We found that the Psm receptor expression (FPR) in intestinal tissue was low compared to DRG which does suggest that Psms might preferentially target afferent neurons. It is also known that enteric neurons are smaller which may, consistent with our cell size analysis, contribute to their lower susceptibility. Alternatively, it could be that microglia which reside in close association with neurons in enteric ganglia, prevent the interaction with neuromodulatory components in SSA. In contrast, afferent fibers are rather unprotected from bacterial mediators also because they constitute mostly thinly or unmyelinated fibers. Interestingly, enteric glia have been reported to express proteolipid protein 1 (PLP-1) and myelin basic protein (MBP), markers of myelinating glia (Rao et al., 2015), and it could therefore be that the myelin-like properties of glia indeed protects enteric neurons. Future studies may aim to further investigate this difference because it could indicate a selective activity of SSA towards unmyelinated fibres which constitute nociceptors.

Smooth muscle cells

In the motility recording experiments, we observed a profound decrease of contraction amplitude but not frequency by SSA which is indicative of a modulation of muscle contractility (chapter IV, Figure 6, p 140). This inhibitory activity was apparent at a concentration of 5 % and dependent on AgrA-regulated mediators. SSA from the AgrA-deficient mutant did not reduce contraction amplitude. In fact, it tended to be higher than during vehicle application which suggests that other mediators particularly those that are inhibited by quorum sensing (peptidoglycans, chapter I) have an opposing activity towards muscle cells. With regard to the mediators involved in this inhibition, we have found that those present in JE2 and Newman (Hla and potentially Psm) are likely to play a role in the inhibitory activity during SSA application. The inhibition caused by JE2 was more sustained which indicates that other factors contribute as well. Interestingly, Hla has been used to in earlier studies of muscle physiology because it makes the muscle cell membrane more permeable for ions such as calcium (Crichton and Smith, 1991; Kitazawa et al., 1991; Nishimura et al., 1988) while simultaneously retaining responses to receptor agonists which argues against a genuine cytotoxicity of α-haemolysin pores. In contrast, it suggests that the decrease of contractility may result from an insufficient removal of calcium from the cytosol of muscle cells. Calcium is required for the induction of contractions in smooth muscle

(Kuo and Ehrlich, 2015) and failing to remove calcium would result in spastic contraction or potentially desensitisation of the contractile apparatus. The calcium sensitivity can also be changed via G protein-coupled receptor signalling and thus, Psm-mediated FPR signalling could be involved in the decrease of contraction amplitude (Nishimura et al., 1988). In addition, Hla pores are permeable for ATP which is also necessary for smooth muscle contractions.

Epithelial cells

The data presented in chapter II suggests that epithelial cells are resistant and unresponsive to mediators in SSA when they are applied to the apical surface. Not only did luminal application of undiluted SSA not recapitulate the effect of serosal SSA with regard to the degree of excitation and inhibition of afferent nerves, application of SSA to the luminal chamber in Ussing Chamber experiments did also not profoundly affect SCC. This is consistent with the barrier function of the epithelium.

When applied to the serosal side, SSA exerted a strong prosecretory effect that was significantly reduced when SSA devoid of Hla were applied (chapter IV). This is in line with ADAM10 being expressed at high levels in stem cells and non-secretory epithelial cells (Jones et al., 2016; Tsai et al., 2014) and also with the reported bias of Hla for the basolateral side of a polarised epithelium (Bell and Quinton, 1992). The mechanism underlying Hla-induced secretion in intestinal epithelial cells has not been unravelled yet. Secretory diarrhoea during *E. coli* and *Vibrio* infection are mediated by activation of intracellular signalling pathways that increase cGMP and cAMP. These directly modulate the activity of apical channels involved in ion secretion. In contrast, commensal bacteria, *Shigella* and *Salmonella* induce secretion from enterocytes secondarily to activating the immune system via their receptors, inflammatory mediators (TNF-α and IL-6) increase Ca^{2+} which also increases secretion. A similar mechanism has been shown to be involved in diarrhoea induced by oral gavage of staphylococcal peptidoglycans. Interestingly, this study found that translocation was required and that it was mast cell mediators that contributed to the development of diarrhoea (Feng et al., 2007). The predominant role of Hla and potentially peptidoglycans is in line our finding that the expression level of receptors for other virulence factors of *S. aureus* (FPRs) is low in intestinal tissue and with a limited contribution of Psms to the SSA-induced secretion.

1.4.3 Other factors contributing to sensitivity to SSA

In most of our experiments, we observed a high level of variability. This was particularly prominent in nerve recording experiments with SSA from the Psm- and protease-deficient mutants. This could mean that under conditions of reduced virulence, host factors have a bigger impact on the sensitivity to SSA. Interestingly, it was the inhibitory effect of SSA that was more sensitive to host factors suggesting that inhibition may in fact constitute a defence mechanism of the host (refer to section 2.1, p 177 for a detailed discussion of excitation and inhibition). We have aimed to address some of these factors in our experiments.

Microbiota

The results from our study do not suggest an overwhelming influence of the microbiota on the overall response pattern of intestinal afferents to SSA. We observed the same biphasic pattern to SSA-JE2 in mice from three different animal sources, regardless of whether or not they carried endogenous *Staphylococci* and in tissue with a high (colon) or low (small intestine) bacteria density prior to the experiment (chapter II, chapter III). However, we did not assess the actual composition of the microbiome of our mice, nor do we know whether mice from the Sheffield facility might have carried endogenous *Staphylococci* prior to our assessment of bacterial load. Thus, our findings to not argue against a modulating role of the intestinal microbiota. In fact, we found small differences with regard to each of these factors suggesting that priming of neuronal activity/the immune response by commensal bacteria does indeed contribute to the variability. In future studies, it would be interesting to investigate the effect of SSA on afferent activity in germfree mice, in mice with a defined microbiome composition but intact immune development (e.g. altered Schaedler's flora) or in mice colonised with non-pathogenic *Staphylococci* to investigate a potential contribution of the microbiome in a targeted approach.

Inflammation and epithelial barrier integrity

We asked whether an acute inflammatory insult altered the response pattern to intraluminally or bath-applied SSA and induced TNBS colitis 21-28 d prior to the nerve recording experiments. Overall, the response patterns of intestinal afferents to SSA in treated compared to control mice (chapter III) were not qualitatively different but the level of SSA-induced inhibition of spontaneous discharge and mechanosensitivity tended to be smaller in treated compared to healthy animals. This finding was unexpected because inflammation causes an increase of intestinal permeability and it is known that colonic nerves maintain hypersensitivity until 28 d after TNBS colitis. The reduced sensitivity to SSA

could be the result of infiltration with immune cells (Campaniello et al., 2017; Wirtz et al., 2017) that are able to inactivate neuromodulatory substances. It has been shown that in fibroblasts activation of stress-induced kinases contributes to insensitivity to Hla via regulation of ADAM10. Inhibition of these pathways increases sensitivity to Hla (Hoven et al., 2015). Inflammation constitutes a profound stressor and thus, these pathways may contribute to a decreases sensitivity of nerves after TNBS colitis. In future experiments, the response to SSA should be assessed in acutely inflamed tissue to better understand the effect of inflammation on sensitivity to SSA.

Host genetic background

We have performed Ussing Chamber experiments in guinea pig and mouse mucosa-submucosa preparations from distal small intestine. In both species, serosal application of SSA-JE2 had a strong prosecretory effect which suggest that they are both susceptible to the same mediators. However, we observed an early response pattern within the first 3 min of application in preparations from guinea pig (chapter II, IV) but not in mice which suggests that mice are resistant to components of SSA involved in this response. Mice have been shown to be resistant to the Pantom-Valentine leucocidin (PVL) because of a mutation in the PVL receptor C5aR (Labandeira-Rey et al., 2007; Spaan et al., 2017). Alternatively, the presence of two distinct response patterns in guinea pig but not in mice could be related to the size of the animals and thus, the sequential activity of different mediators in guinea pig whereas in mice they act simultaneously. Differences have not only been observed between animal species but also with regard to mouse strains. For example, Balb/c and A/J mice are more sensitive to *S. aureus* infection than C57Bl/6 animals because of a dominating T_H2 response which impairs successful removal of *S. aureus* (Von Köckritz-Blickwede et al., 2008; Thänert et al., 2017). We have so far not addressed strain specificity.

Lipid metabolism

Lipid metabolism is emerging as an important variable during *S. aureus* infections. Apolipoprotein B (Apo B) which is associated with cholesterol-rich low and very low density lipoproteins (LDL, VLDL) is more abundant in mice resistant to *S. aureus* and ApoB48, a splice variant of ApoB produced only in enterocytes, also alleviates *S. aureus* pathology in a mouse model of skin and soft tissue infection (Peterson et al., 2008; Thänert et al., 2017). On the contrary, genetic or pharmacological reduction of LDL increases the severity of *S. aureus* infection and hypolipoproteinemia increases the risk of bacterial infection in patients (Castleman et al., 2018; Elmore et al., 2015). These findings however do not suggest that pathologically high levels of cholesterol as seen in high fat diets could be beneficial during *S. aureus*

infections. Mechanistically, both apolipoproteins have been shown to bind the auto-inducing peptides (AIP) that induce Agr signalling at high population density and thus, antagonise quorum sensing-induced virulence factor secretion. Pre-incubation of supernatants with apolipoproteins may therefore have a similar effect as AgrA knock-out with regard to modulation of afferent nerve activity. In humans, antagonising AgrA-regulated gene expression through ApoBs was associated with better clinical outcome and promotion of bacterial clearance (Sully et al., 2014). Serum lipoproteins have also been shown to bind and neutralise neuromodulatory substances such as Psms (Surewaard et al., 2012) and this might be another explanation for their beneficial effects during infection. We have not assessed metabolic parameters in our mice, nor did we record body weight or fat mass but these might be important parameters that contributed to the large variability of the nerve recording experiments.

It was not the scope of our study to systematically investigate the effects of host factors on susceptibility to SSA and therefore, our data does not allow to draw final conclusions as to whether these factors contribute to the variability that we observed in our studies. It will be important to assess these factors when it is clear how the SSA-induced neuromodulation relates to pathology in *S. aureus* infections.

2 Physiological interpretation of pathogen-mediated alteration of neuronal activity and intestinal function

2.1 Is excitation or inhibition pathogenic?

In our study, we have found that different strains of *S. aureus* vary with regard to their neuromodulatory properties (chapter IV, Figure 3, p 128). The data from the strain analysis was largely in line with the predicted outcome based on published analyses of the bacterial genome (Chapman et al., 2017b). The Newman and the JE2 strain both profoundly increased intestinal afferent nerve activity and produce large abundances of α-haemolysin which we found contributed most to SSA-induced excitation. JE2, in contrast to Newman, also produces Pvl which plays an important role during lung infections particularly because it releases IL-1β from resident macrophages. Interestingly, Perret et al. have shown that the Pvl-induced IL-1β secretion is profoundly potentiated by Psms (Perret et al., 2012). Although it is known that murine cells are generally less susceptible to Pvl because of a mutation in its receptor C5a (Spaan et al., 2013), this synergistic effect between Pvl and other toxins may contribute to the marked difference between SSA-JE2 and -Newman during long-term application of SSA. While SSA-JE2 were found to inhibit nerve activity, SSA-Newman did not. Newman produces more

haemolysins and leucocidins with a potential excitatory effect and less proteases which may reduce tissue penetration of neuromodulatory substances (Chapman et al. 2017). The SH1000 strain displayed an intermediate phenotype with regard to excitation and inhibition which is in line with analyses of gene expression and a reduced severity inflammation in a tail infection model in susceptible hosts (Horsburgh et al., 2002; Thänert et al., 2017).

We found that both highly virulent strains (JE2 and Newman) increased nerve activity and cell membrane permeability but Newman did not inhibit despite secreting large amounts of immunomodulatory proteins, exoenzymes and cytolysins (Baba et al., 2008; Bae et al., 2004; Chapman et al., 2017b). This suggests that the high degree of excitation is related to exaggerated virulence. Indeed, we found that the SH1000 strain of *S. aureus* which is less virulent than JE2 and Newman, induced less excitation and supernatants from bacteria isolated from the endogenous microbiota only exerted a small excitatory effect. Others that have investigated a potential link between neuronal activation and bacteria found that species of commensal bacteria such as *Clostridium ramosum* and *Bacillus ovatus* were able to activate neurons to a similar degree as *S. aureus* (Blake et al., 2018; Yissachar et al., 2017) which suggests that these may also have pathogenic potential. Indeed, *C. ramosum* and *B. ovatus* have been described as commensals linked to obesity and IBD respectively (Saitoh et al., 2002; Woting et al., 2014). Interestingly, application of these bacteria to an intestinal organ culture system also decreased the expression of neuropeptides and neuropeptide receptors in intestinal tissue which indicates that nerve inhibition may constitute an adaptive response to some bacteria. It is less likely to be linked to virulence because in our study, the JE2 and the SH1000 but not the Newman strain inhibited nerve activity.

Future studies should translate these findings and assess whether the observed distinct effects of SSA with regard to neuromodulation translate into measurable differences of *S. aureus* pathogenesis induced by different strains. In these studies, the effect of bacteria on neurons and the immune system need to be addressed in parallel to better understand the time course of neuro- and immunomodulation. It would also be interesting to apply supernatants from commensal bacteria that were shown to activate sensory neurons to afferent nerves.

2.2 Function and physiological relevance

Intestinal sensory signalling plays important roles in sensation as well as in the regulation of spinal reflexes (chapter I, section 2.1.3, p 45). These in include urge, fullness, discomfort and pain but also behaviour, food intake and immune functions. We found that mediators released by *S. aureus* had profound effects on intestinal afferent activity and mechanosensitivity and will discuss possible implications for gut-brain signalling during pathogenic infection.

2.2.1 Vagal reflex

In our studies, we recorded the activity of afferent nerves innervating the small intestine. These constitute fibers originating from nodose and dorsal root ganglia. The vagus nerve is distinct compared to other cranial nerves because it contains a large percentage of sensory (afferent) but fewer motor (efferent) fibers. Through vagal reflex circuits and antidromic pathways, activation of vagal signalling influences most visceral organs including the GI tract and spleen as well as liver, stomach and heart via the release of acetylcholine (Breit et al., 2018; Pavlov and Tracey, 2012).

Vagal reflex circuits have an essential role in sensing and eradicating oral pathogens. Emesis is important to expel harmful substances such as bacteria before they passage and translocate into the gut wall where they could cause severe long-term damage. The pathological features of vomiting are complex, require coordination by the PNS and include hyper-salivation, inhibition of gastric motility, relaxation of oesophageal sphincters, retro-peristalsis in the upper gut etc. (Babic and Browning, 2014). Emesis as well as -associated symptoms (tachycardia, sweating) are frequently observed during *S. aureus* food poisoning and this may also account for the abrupt onset and self-limiting progression of the disease (Kadariya et al., 2014). We found that most *S. aureus* strains increased intestinal afferent nerve activity, and if these are vagal afferents this may also induce a vomiting reflex. However, excitation was dependent on Hla in our study which has not previously been studied as an emetic toxin in *S. aureus*. On the contrary, vomiting has been associated with the production of staphylococcal enterotoxins (SE). Exactly how these induce emesis is not fully understood yet but it is suggested that SE induce serotonin release from resident mast cells, neurons or EEC which in turn activates vagal fibers (Hu et al., 2014). A direct interaction has also been suggested for SE-induced emesis but it is not clear whether this involves the T cell receptor which is involved in sensing of SE by immune cells or other, unidentified receptors. Our finding that some strains of *S. aureus* inhibited afferent nerve firing could mean that these are able to prevent the emetic response leading to their passage into the GI tract.

would be particularly true for mutants lacking α-haemolysin and AgrA as well as endogenous Staphylococci which indeed have been found in invading/chronic disease (Laabei et al., 2015). However, circulating 5-HT and other mediators can also act on the brainstem to cause emesis (Mayer 2011, Rhee et al., 2009) which would bypass these peripheral mechanisms.

Vagus nerve stimulation has been also been found to exert anti-inflammatory effects on peripheral tissues ("Cholinergic anti-inflammatory pathway", CAP). For example, TNF-α release from splenic and intestinal macrophages can be inhibited through vagal acetylcholine (Bonaz et al., 2017). At first sight, it appears counter-intuitively as to why these pathways should become activated during acute inflammation especially bacterial infections which require efficient phagocytosis. Stimulation of CAP could aim at limiting the innate immune response and enable the development of a more targeted adaptive response which might be crucial for host survival and immune memory. However, an overwhelming activation could also result in an increase of the bacterial burden and thus prolong disease duration (Dalli et al., 2017). In this regard, our finding that nerve activity was inhibited during USA300 (JE2) application, if it were to lead to a decrease in the CAP, may be interpreted as stimulation of phagocytic activity once the acute threat has passed.

2.2.2 Sensation

We found that mediators from *S. aureus* activated intestinal afferents which constitute predominantly unmyelinated and thinly myelinated C- and Aδ-fibers and express markers of nociceptive neurons such as TRPV1 and CGRP. This suggests that activation of these neurons can induce the pain perception. Indeed, a close association between *S. aureus* and pain sensation has emerged from previous studies. Intraplantar injection of live and heat-killed *S. aureus*, formyl-peptides, Hla, Psm or γ-haemolysin induces spontaneous pain which is independent from the inflammatory pain during later stages of the inflammation and not mediated by an activation of the immune system (Blake et al., 2018; Chiu et al., 2013). Instead, these studies show sensory neurons are directly activated by bacteria and its mediators. The purpose of spontaneous pain induced by bacteria can so far only be speculated. Somatic pain in response to heat or mechanical stimuli results in acute withdrawal of the injured tissue from the pain-causing stimulus. In the case of bacteria and particularly in the intestine, these reflexes cannot enable to escape and therefore, pain sensation may be an indicator to signal that "something is wrong". In addition, nociceptor activation has been shown to modulate immune responses and increase blood flow at the site of infection.

In addition to neurons that classify as nociceptors, the small intestine is also innervated by a second class of small to medium sized neurons that do not express neuropeptides and little TRPV1 and are involved in itch sensation. These neurons abundantly express purinergic receptor P2X3 and members of the mas-related G protein-coupled receptor (MRGPR) family (Hockley et al., 2018) and tend to be larger than nociceptive neurons. They are activated by ligands of the MRGPRs including BAM8-22, compound 48/80 and chloroquine and this causes itch (Han et al., 2013; Solinski et al., 2014; Usoskin et al., 2014). Itch can also be induced though histamine which is released from mast cells upon activation with IgE for example. Because mast cells are located in close proximity to intestinal neurons and histamine is known to contribute to afferent nerve sensitisation (Deiteren et al., 2014; Song et al., 2015), this population of neurons might be particularly interesting in future studies. Phenol-soluble modulins which contributed to nerve inhibition in our experiments, appear to be closely linked to itch sensation. They have been shown to induce mast cell degranulation via activation of FPR2 (Hodille et al., 2016; Nakamura et al., 2013) and may, based on their structural properties, also directly activate MRGPs. The notion that Psms are involved in itch rather than pain sensation is also supported by the observation that Psms were dispensable for spontaneous pain during *S. aureus* skin infection (Blake et al., 2018) and that *Staphylococcus aureus* is frequently detected in patients with atopic dermatitis and psoriasis (Kim et al., 2018). The potential function of "itch-in-the-gut" remains to be identified but it is possible that abdominal discomfort is associated with this sensation.

2.2.3 Behaviour

In addition to the thalamo-cortical projections that are involved in conscious sensations, intestinal sensory neurons are also connected to CNS structures regulating behaviour. Activation of these pathways by mediators of *S. aureus* could contribute to sickness behaviour including fatigue, reduced activity, social isolation and anxiety (Griton and Konsman, 2018). In studies where mice were injected with the endotoxin LPS into the peritoneum, it was found that these animals displayed a depressive phenotype as measured by social interaction 29 d after injection which was reduced in vagotomised animals (Bluthé et al., 1994; Konsman et al., 2000; Luheshi et al., 2000; Zielinski et al., 2013). Although these studies could be interpreted as evidence for a direct LPS-induced activation of vagal signalling, it is also possible that LPS injection causes the release of cytokines from resident immune cells which in turn activate afferent nerves, c-Fos expression in the brain and induce sickness behaviour (Bluthe et al., 1996; Campaniello et al., 2016; Dantzer, 2009; Konsman et al., 2000). However, others have reported

similar changes already 7-8 hours after treatment of mice with pathogenic bacteria. For example, oral administration of *Campylobacter jejuni* increases expression of a marker for neuronal activation (c-Fos) in brain regions involved in the control of behaviour as well as anxiety in control but not in vagotomised animals (Goehler et al., 2008; Lyte et al., 1998). Similarly, gavage of *Citrobacter rodentium* increases anxiety and c-Fos expression in the brain at early stages of the infection where plasma cytokine levels are not affected (Lyte et al., 2006). Thus, these studies suggest that pathogenic bacteria via direct interaction with vagal and/or spinal nerves modulate brain activity.

More recently, bacteria-mediated modulation of behaviour has been investigated with regard to commensal and probiotic bacteria (Cryan and Dinan, 2012). Ingestion of different strains of *Lactobacillus* and *Bifidobacterium* as well as faecal transplantation of microbiota have profound effects on mood and anxiety and sickness behaviour (Bravo et al., 2011; D'Mello et al., 2015; Kelly et al., 2016; Liang et al., 2015; Messaoudi et al., 2011). In these studies, probiotic bacteria are administered to mice or human participants for an extended period of time (more than 10 d) and this has beneficial effects on disease-associated behaviours such as anxiety but also sensations. Although some of these bacteria have been shown to produce substances with a potential neuromodulatory function such as GABA etc., it remains to be determined whether probiotic treatment alters brain function directly through an interaction between the bacteria and neurons or secondarily through alterations of the inflammatory milieu and metabolism (Cohen et al., 2017; Hyland and Cryan, 2010; Pokusaeva et al., 2017). To investigate the former possibility, it would be possible to compare the effects of probiotics in mice where receptor proteins are abolished selectively in sensory neurons. However, it is still unclear exactly which receptors are involved and therefore, studies performed in animals lacking (subtypes) of immune cells or blocking their activation might be an alternative approach.

2.2.4 Modulation of food intake

Other hallmark symptoms of gastroenteritis are anorexia and nausea. As described in chapter I (2.1.3, p 45), food intake can be regulated by sensory nerves via hypothalamic projections. In health, this is important for the postprandial suppression of food intake for example. Our finding that bacterial mediators modulate activity of those sensory nerves suggests that bacterial mediators may activate aspects of satiety signalling such as POMC (proopiomelanocortin) neurons in the arcuate nucleus of the paraventricular hypothalamus that would lead to anorexia. This is in line with previous studies showing that LPS-induced reduction of food intake can be prevented via vagotomy (Bretdibat et al.,

1995). More recent studies confirmed that neural mechanisms are involved in bacteria-induced sickness behaviour including food intake but from those studies it is not clear whether it is bacteria themselves or the cytokines produced by immune activation are involved in the anorectic response (Harrison et al., 2009; Rao et al., 2017b). Interestingly, the study by Rao et al. (2017) also suggests that some pathogenic bacteria might also increase food intake for their own benefit and to prevent starvation. They found that pathogenic but not non-pathogenic strains of *Salmonella thyphimirium* increased food intake i.e. repressed sickness-induced anorexia via the vagus nerve to promote the survival of the host and its own transmission. Our finding that some strains of *S. aureus* both excited and inhibited afferent nerves but others only increased spontaneous discharge might be related to this dual effect of bacteria on food intake. It is currently unknown whether these strains have different effects on food intake.

2.2.5 Immune response

It is becoming increasingly clear that the nervous and the immune system are closely related. Cytokines released by the immune system have a sensitising effect on neurons and we are also beginning to understand how neurons modulate the inflammatory response through the release of transmitters at their peripheral and central terminals. In this regard, sensory neurons and particularly those that secrete neuropeptides i.e. nociceptors play an important role in communicating peripheral inflammatory signals induced by bacterial mediators and cytokines to the brain which cannot cross the blood brain barrier themselves. Ablation of these neurons has profound effects on the host response to microbial infection and also on immunity (Baral et al., 2018; Chiu et al., 2013; Talbot et al., 2015).

We found that excitation of intestinal afferents which constitute mostly transmitter-releasing neurons, was a common feature of all SSA except for Hla-deficient bacteria (SSA-Hla$^-$, SSA-AgrA$^-$). Depolarisation and activation of peripheral terminals of sensory nerves induces the release of neurotransmitters such as CGRP, SP and ATP from those nerve terminals but also from central processes which can modulate immunity. Because Hla is produced by *S. aureus* strains that cause lethal infections and bacteraemia, it is possible that an acute pain sensation is indicative of (hyper-) activation of the immune system aiming at eradicating the intruder at any cost. This hypothesis is consistent with studies showing that acute lethality during infection with high doses of *S. aureus* is reduced when it does not produce virulence factors that are implicated in pain sensation (Heyer et al., 2002; Rauch et al., 2012; Rudkin et al., 2012). Because successful eradication of *S. aureus* overload will require phagocytosis by innate immune cells such as macrophages and neutrophils, this is likely to involve a

strong T_H1/T_H17 response (Bröker et al., 2016). Interestingly, colonisation of intestinal organ cultures with T_H17-inducing bacteria also increases the expression of neuropeptides (Yissachar et al., 2017).
In contrast to these lethal infection studies, it has been found that human isolates that produce large amounts of toxins are associated with a mild disease progression as it is frequently observed during skin and soft tissue infections (Cheung et al., 2014; Laabei et al., 2015; Qi et al., 2016). This could be associated with an enhanced recognition and the development of counter-regulatory responses to prevent over-activation of the immune system. Indeed, phenol-soluble modulins have been shown to induce regulatory T_{reg} cells that would be able to control inflammation (Armbruster et al., 2016a, 2016b; Schreiner et al., 2013). We have found that Psms inhibit intestinal afferent activity and with this regard, it is striking that Yissachar et al. (2017) found that T_{reg}-inducing bacteria reduce neuropeptide expression in their intestinal organ culture model. In vitro, these bacteria as well as Psms from *S. aureus* were found to acutely increase neuronal excitability. This suggests that both, recognition of the intruder as well as induction of a regulatory response are reflected in afferent nerve activity as excitation and inhibition respectively. We hypothesise that the level of excitation caused by T_{reg}-inducing bacteria might be lower than under T_H1 conditions and is not associated with spontaneous pain.
Non-haemolytic and Agr-deficient strains which would not cause excitation in our experiments, are more frequently recovered from chronic infections in peritoneum, lung, wounds and during antibiotic treatment (Kumar et al., 2017; Rauch et al., 2012; Schwan et al., 2003; Sugiyama et al., 2009). In a model of chronic pneumonia, Psm expression was increased regardless of a decreased Agr activity (Chaffin et al., 2012) which supports the hypothesis that inhibition may indeed be linked to a regulatory response which enables bacterial survival.

Others have used genetic or chemical ablation to study neural control of the immune system. Initially, Chiu et al. (2013) found that ablation of $Na_V1.8^+$ neurons increases neutrophil infiltration of skin tissue acutely after injection of *S. aureus* into the hind paw. In addition, chemical and DTA-induced ablation of nociceptive fibers increases the mobility and activity of neutrophils which was interpreted as a nociceptor-mediated inhibition of neutrophil in animals with intact sensory signalling (Baral et al., 2018). Because silencing of these neurons would interfere with both the excitatory and inhibitory response, it is difficult to relate the findings to our data. If the excitatory component which appears to be indicating pathogen recognition and eradication, was predominantly affected, we would expect that this results in an increase of bacteria load due to ineffective eradication. This however has not been observed and therefore, it is possible that denervation regulates neutrophils indirectly by decreasing the T_{reg} response.

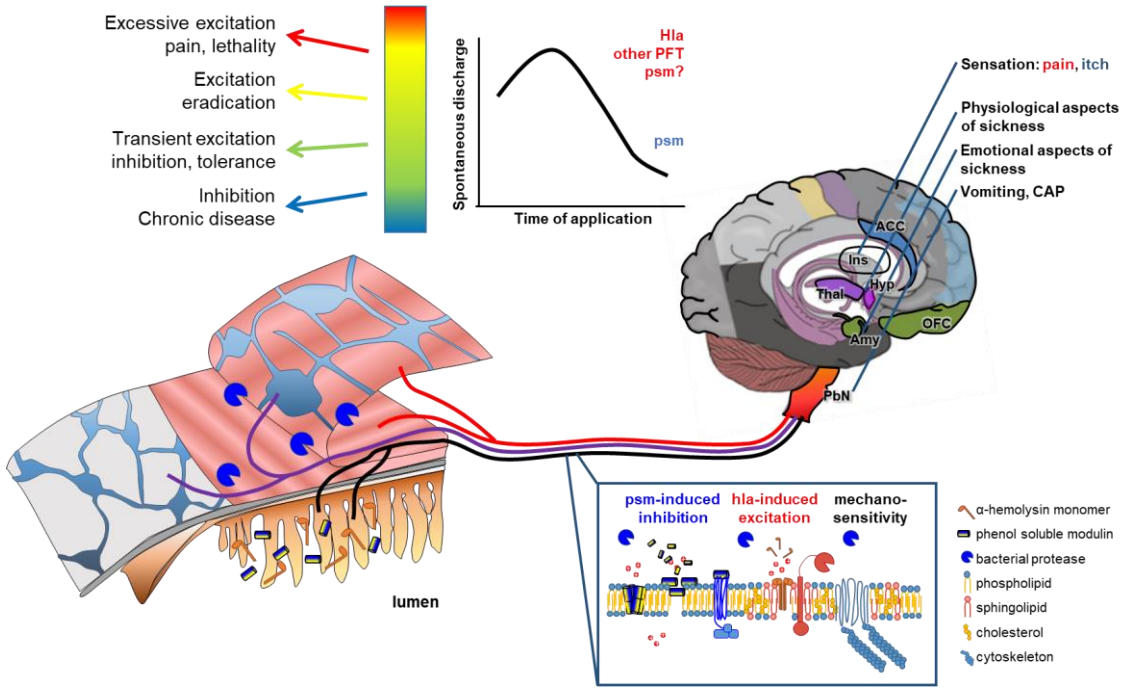


Figure 1: Hypothetical model of SSA-induced changes of neuronal activity and physiological consequences of neuromodulation. Pore-formation though multimerisation of α-hemolysis (Hla) close to its receptor ADAM10 increases nerve activity whereas Psm-induced disruption of the cell membrane or intracellular signaling through FPRs contributes to inhibition. Strain differences with regard to the degree of excitation and inhibition suggest that those are associated with distinct outcomes of infection. In addition, activation and inhibition of extrinsic sensory signaling will affect reflex behaviors and central circuits regulating sickness behavior and sensation. Refer to the text for more details

ACC, Anterior cingulate cortex; Amy, amygdala; CAP, cholinergic anti-inflammatory pathway; FPR, formyl peptide receptor; Hyp, hypothalamus; Ins, insula; OFC, orbitofrontal cortex; PbN, parabrachial nucleus; PFT, pore-forming toxin; Psm, phenol-soluble modulin; Thal, thalamus

In summary, these findings suggest that neuromodulation is an important contributor to regulating the response to bacteria and may enable to distinguish between the "good" and "bad" bacteria. We hypothesise that bacteria such as foodborne pathogens that can penetrate the intestinal barrier induce a high degree of excitation which is involved in spontaneous pain and a T_H1/T_H17 response aiming at eradication. On the contrary, a transient excitation and/or inhibition of nerve activity induced by commensal bacteria possibly induces tolerance. Pathogens may abuse this pathway to induce ignorance of the infection leading to chronic illness. This pathway could potentially involved in the transition from acute to chronic disease and could explain why chronic inflammatory diseases such as IBS and IBD often develop after gastroenteritis. It seems possible that a direct neuron-bacteria interaction during the acute phase predisposes an individual for IBS. This possible link between neuronal activation and the induction of an immune response should be addressed in future research.

2.3 Effect of SSA on intestinal function

We found that mediators from *S. aureus* also affected intestinal secretion and motility. These effects, to our surprise, were not mediated by modulation of neuronal activity which suggested that enteric neurons were less susceptible to SSA than intestinal afferent neurons (chapter II). Alternatively, effects on neuronal activity might be masked by the mediators inducing secretion. With regard to the physiological consequences of our findings, they are in line with *S. aureus* food poisoning being associated with diarrhoea. An increase of secretion as well as a decrease of motility would decrease the time that ingesta is in contact with the intestinal epithelium. The mechanisms underlying staphylococcal diarrhoea are not understood yet nor have previous studies specifically looked at the effect of individual staphylococcal toxins on the intestinal epithelium. These are likely to be involved in the prosecretory activity because it was significantly reduced in the AgrA mutant. Clinical evidence also supports this idea. Most isolates that were associated with food poisoning were shown to produce a number of these toxins and only some are associated with diarrhoea which suggests that it is relevant which toxins are produced (Argudín et al., 2010; Wattinger et al., 2012). Although toxin-producing strains of *Staphylococcus aureus* can be frequently recovered from foods such as meat, eggs, pastries and milk products, the number of hospitalised patients and severe infectious gastroenteritis is relatively low but it is suggested that *Staphylococcus aureus* plays a big role patients that do not seek medical advice(Argudín et al., 2010; Kadariya et al., 2014).

The strong inhibitory effect of the USA300 strain *S. aureus* in our study could also be related to dysmotility pattern in Salmonellosis patients. Prolonged periods of quiescence have been described in these patients indicating that bacteria other than *S. aureus* can inhibit contractile activity of intestinal muscle (Accarino et al., 1993). With regard to the role of this inhibition during infection, it may be speculated that it is for the benefit of the bacteria to reduce contractions and enables to invade the tissue. In contrast, by stimulating ion, water and potentially mucus secretion these bacteria might be able to reduce the number of competing commensal bacteria.

3 Conclusions and perspectives

3.1 Why study pathogenic bacteria?

Dysregulation of sensory and inflammatory signalling as well as intestinal function are observed in many chronic diseases such as inflammatory bowel disease, irritable bowel syndrome, obesity and diseases of the central nervous system. These diseases constitute major concerns for human health, their prevalence has increased over the years and they have significant socio-economic impact. Regardless of the enormous efforts and research, our understanding about the pathogenesis of these diseases is still limited (Bennet et al., 2015; Mulders et al., 2018; Rhee et al., 2009). In recent years, the composition of the microbiome has been discussed as a major factor associated with these diseases. It has been found that the microbiome of patient cohorts differs from healthy controls (Tap et al., 2017; Turnbaugh et al., 2006; Yarandi et al., 2016). The factors that are associated with microbiome dysbiosis include stress, the use of xenobiotics and diet and these are also related to the development of those chronic diseases (David et al., 2014b; Foster et al., 2017; Rajpal et al., 2015). Although it is not clear yet what exactly constitutes a "diseased microbiome", many studies find an increased abundance of potentially pathogenic bacteria (Duvallet et al., 2017). Because of the strong link between dysbiosis, pathogens and chronic diseases, it is highly relevant to study how pathogenic bacteria interact with the host.

For these reasons, this thesis investigated the potential of soluble mediators released from the opportunistic pathogen *S. aureus* to modulate visceral sensitivity and intestinal function. Our data shows that factors contributing to the success of *S. aureus* as a pathogen cause profound changes of sensory neurons and intestinal afferent activity. This does not only add bacterial toxins to the wide range of neuromodulatory compounds but also provides a new insight into host-bacteria communication during acute infection. Traditionally, it has been thought that it is the immune system that is responsible for pathogen detection and that neuronal aspects of inflammation (calor, dolor, sickness behaviour) depend on the release of cytokines. However, neuronal detection may constitute a fast alert signal aiming to prepare the host for the inflammatory response and contribute to successful eradication of the intruder as well as survival of the host. Furthermore, neuronal detection might have long term consequences. Acute stimuli can induce neuronal remodelling such as neuronal sprouting, changes of receptor and ion channel expression which are thought to result in changes of neuronal excitability and lead to disease (Brierley and Linden, 2014).

3.2 What is the relevance of serosal and apical application?

Commensal bacteria colonising the intestine are contained within the lumen by the intestinal epithelium and a layer of mucus reduces the number of bacteria that can attach to the epithelium or translocate into deeper layers of the gut wall (Bäckhed et al., 2004b; Liévin-Le Moal and Servin, 2006). We found that these defence mechanisms also affected the response pattern to SSA. A biphasic response pattern of afferent nerves was observed at a concentration of 20 % during bath application whereas intraluminal application of undiluted (100 %) SSA increased spontaneous nerve activity with no inhibition. When the epithelium was disrupted however, a small degree of inhibition became apparent with luminal application. In addition, it was only serosal, but not apical, application of SSA in Ussing Chambers that increased secretion. These findings emphasise the barrier function of the epithelium and the importance of detecting bacteria that were able to overcome the intestinal barrier which is a feature predominantly of pathogenic bacteria. In this regard, *S. aureus* has been shown to asymptomatically colonise the GI tract (Misawa et al., 2015) but also persist in epithelial cells as well as phagocytes and erupt from these cells after proliferation which would result in a high local concentration of bacterial mediators (Jubrail et al., 2016; Strobel et al., 2016). In addition, bath application may also reflect how infections at other sites of the body and bacteraemia can affect sensory signalling and intestinal function.

To our surprise, we found that acute inflammation induced by TNBS instillation into the colon tended to reduce the sensitivity of intestinal afferent nerves to bacterial supernatants. TNBS instillation into the colon causes acute inflammation which is associated with increases of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) and hypersensitivity of colonic afferents. While the inflammation resolves by 21 d, hypersensitivity in colonic afferents is maintained (Hughes et al., 2009). For the first time, we have shown that colonic inflammation does not induce hypersensitivity in small intestinal afferents but reduces the response to SSA. The prominent decrease of spontaneous firing that we observed in control animals in the presence of bath-applied SSA as well as the reduction of mechanosensitivity were significantly attenuated in some TNBS-treated animals. We have not investigated the concentration of pro- and anti-inflammatory cytokines or epithelial permeability and secretion but it would be interesting to investigate whether these parameters contribute to the reduced sensitivity to SSA in these animals. Future studies should also look at different time points after TNBS administration and use mice deficient of immune components to better understand the impact of the host immune status on the response to bacterial mediators.

3.3 What are the neuromodulatory substances produced by *Staphylococcus aureus*?

We used a microbiological approach to dissect out which mediators were involved in the neuromodulatory effects of SSA during bath application. Two types of toxins mediated different aspects of the biphasic afferent response pattern of intestinal afferent neurons. The pore-forming toxin α-haemolysin contributed to excitation whereas phenol-soluble modulins were involved in the inhibitory effect of SSA. This is in contrast to capsaicin and resiniferatoxin (TRPV1 agonists) for example that reduce neuronal activity at high concentrations and have been used clinically to ablate chronic pain conditions. A major side effect of these compounds is the severe pain during application and the necessity of repeated treatment. With this in mind, our findings that the Hla-deficient Psm-producing mutant caused inhibition without excitation and that Psms were predominantly involved in inhibition suggests that Psms might potentially provide a new treatment option or tool to reduce sensory signalling to the brain. It remains to be investigated whether Psms target distinct cell types and subsets of neurons, particularly at low concentrations. We found that the prosecretory activity of SSA as well as the inhibitory effect of SSA on motility are unlikely to involve modulation of enteric neuronal activity which suggests a selectivity of SSA towards sensory neurons. These findings however also indicate that diarrhoea and inhibition of muscle contractility might be side effects of such a potential therapeutic Psm application. Others also find that Psms are particularly toxic to neutrophils. Therefore, the duration of neuronal desensitisation versus effects on intestinal function and immune cells should be established in future studies.

In our study, the effects of SSA on afferent discharge as well as intestinal function were completely dependent on mediators released at high population density. QS activates the transcription regulator AgrA and this increases the production of secretory proteins. This would be a concern with regard to infection and also in the intestine because one would anticipate the absolute abundance of *S. aureus* to be small compared to other bacteria. In addition, the immune response (ROS) and metabolic signals decrease the transcriptional activity of AgrA (Kavanaugh and Horswill, 2016). However, studies using *S. aureus* knock-out strains have shown that these secreted virulence factors play an important role during infection and are particularly associated with disease severity (Otto, 2013; Peraro and van der Goot, 2016). Therefore, our findings are highly relevant not only from a physiological but also from a microbiological point of view. Here, the effect of bacterial compounds on neurons has so far not been taken into consideration.

3.4 What are the consequences of neuromodulation by bacteria?

In our studies, we have found that strains that differ with regard to their virulence potential also exerted different effects on small intestinal afferent firing suggesting that the response profile of afferent nerves is linked to (patho)- physiological consequences during infection. Investigating this link would need an *in-vivo* approach which was beyond the scope of this thesis. Future studies may attempt to mimic intraluminal or bath application of SSA via oral gavage or intraperitoneal injection respectively and determine survival of the animal, potential pain or itch responses, food intake, behaviour, effects on the immune response and most importantly sensitivity of afferent nerves. We would anticipate that injection of supernatants induces more profound physiological responses than gavage and also that the response to supernatants from different strains/mutants of bacteria would be different. Importantly, responses are likely to occur within a short time period independent of the immune response. Because bacteria themselves are highly immunogenic, supernatants rather than live bacteria should be used in initial studies.

Supernatants from both of the highly pathogenic strains (USA300, Newman) but not SH1000, endogenous bacteria or USA300 mutants devoid of AgrA or Hla, exhibited a strong excitatory response. Therefore, we hypothesise that the degree of excitation might enable to distinguish pathogenic and non-pathogenic strains and would expect to observe differences between these strains in hypothetical in-vivo experiments. For example, supernatants from Newman and USA300 might induce a pain response and acute diarrhoea when injected into the peritoneum and display stronger signs of sickness. These experiments would also be particularly interesting to understand the inhibitory activity of SSA. It is possible that inhibition represents repression of vagal anti-inflammatory reflexes but also that it induces regulatory responses to prevent an overshoot of the immune response which could contribute to bacterial persistence in the host. Thus, determining the activity and expression of marker proteins of different immune cells or bacterial counts would be parameters to compare. Inhibition did not appear to be linked to pathogenicity of the strain because it was not observed in the Newman strain even though both, the USA300 and the Newman strain, had similar activities in a cell-based assay.

To better understand the implications of the effects of bacterial mediators on afferent nerves, it would be interesting to test the neuromodulatory capacity of further strains of *Staphylococcus aureus* but also other bacteria. Here, pathogenic bacteria with documented gut pathophysiology (*Salmonella*,

Citrobacter, *Vibrio*, *Enterobacterium*), commensal bacteria with previously described T cell-inducing properties (*Clostridium ramosusm*, *Bacteroides ovatatus*, segemented filamentous bacteria) and probiotic bacteria with effects on behaviour and mood (*Bifidobacterium longum*, *Lactobacillus rhamnosus*) would be initial targets (Bravo et al., 2011; Chapman et al., 2017b; King et al., 2016; Monteagudo-Mera et al., 2012; Rao et al., 2017b; Savignac et al., 2015; Yissachar et al., 2017).

3.5 How do host factors affect the response to bacteria?

An important observation in our and previous studies that is often disregarded was the variability of responses to bacteria. This variability may be partially attributable to factors that influence supernatant composition but differences between the hosts are also likely to play a major role. For example, the inflammatory status of the animal, its genotype, lipid metabolism etc. may contribute to the differences in sensitivity to SSA. We have shown that the presence of *Staphylococci* in the intestine as well as TNBS-induced colitis dampened to the excitatory and inhibitory effect of bath-applied SSA on spontaneous firing. These factors were associated with subtle changes of sensitivity to SSA but it is likely that the expression of toxin receptor proteins by the host, production of lipoproteins, abundance of immune cells etc. have stronger effects on the susceptibility to SSA-induced neuromodulation (Becker et al., 2014; Elmore et al., 2015; Rautenberg et al., 2011; Surewaard et al., 2012; Vandenesch et al., 2012b). To dissect the contribution of these factors is challenging because they may all be interrelated but it will also increase our understanding as to why *Staphylococcus aureus* causes severe illness in various organs in some individuals whereas it asymptomatically colonises others. In this regard, it should be noted that SSA devoid of Psms continued to inhibit nerve activity in some animals which suggests that either those animals are more susceptible to other factors in SSA or that host factors can inhibit nerve activity as well. Because of the structural similarities of Psms to host defence proteins, it may be interesting to assess whether those have inhibitory activities. To systematically investigate the effect of host factors on susceptibility to SSA, a consistent composition of SSA should be controlled for e.g. using HPLC or mass spectrometry. This is important because bacteria respond to environmental cues and we have found that some batches of SSA had smaller activity in cell-based assays than others.

To further investigate the effect of host factors on the response to SSA, it would be particularly interesting to repeat our experiments in animals lacking a microbiota (germfree animals), animals colonised with a defined or human microbiota. Humanisation of the rodent microbiome via faecal transplantation has been successfully used in other studies and will be a useful tool for future research.

This approach enables to better understand influence factors on the microbiome because genetic and dietary factors can be better controlled in rodents. At the same time, faecal transplantation also transfers human phenotypes associated with changes of the microbiome such as anxiety and visceral hypersensitivity (Crouzet et al., 2013; De Palma et al., 2017).

3.6 Concluding remarks

We set out to perform a "proof-of-principle" study and investigated whether soluble mediators from bacteria were able to directly affect intestinal afferent nerve activity, epithelial function and motility. We indeed found that virulence factors from *S. aureus* modulated these components. Intriguingly, profound effects were only observed when these virulence factors were present at the basolateral side of the intestinal wall which reflects a condition of bacterial translocation or systemic infection.

The virulence factors that contributed to the effects have well-documented roles in the pathogenesis of *S. aureus* but it is not clear yet how exactly they modulate extrinsic sensory neurons, intestinal epithelial cells or enteric neurons. Future studies could aim at unravelling these mechanisms which may unveil a specificity towards distinct cell types or even subpopulations of neurons. This is important for a better understanding of host-bacteria interaction and may be particularly interesting for the mediators that were involved in the inhibitory effect on afferent nerves.
The profound differences between various strains of *S. aureus* were another striking finding that should be further investigated. We hypothesise that these are linked to pathogenicity and symptom severity during infection. It was not within the scope of this PhD to assess these strain differences *in-vivo* but these experiments and analyses of other pathogenic and non-pathogenic bacteria could be promising avenues in future studies.

Overall, our findings show that a direct interaction between bacterial compounds and intestinal structures especially neurons may have important functions during infection and may also induce sustained changes of neuronal activity which are implicated in chronic diseases. So far, these direct interactions have not been considered and it will be exciting to further investigate their contribution to symptoms during infections.

CHAPTER VI

Supplementary methods

This chapter provides detailed information on methods that were used throughout this thesis. The experimental protocols and data analysis are described in the respective results chapters.

All experiments were performed in accordance with the University of Sheffield's Animal Care Committee under UK protocol and project licence following the UK Animals (Scientific Procedures) Act 1986 (chapter II+IV) or with the guidelines of the Animal Ethics Committees of the South Australian Health and Medical Science Institute (SAHMRI) in Adelaide (chapter III).

1 Animals

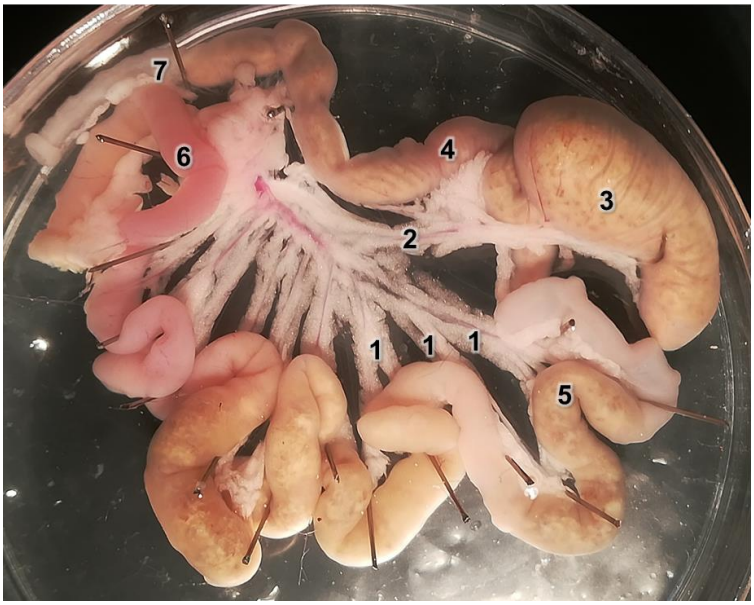
Throughout this study, wild-type adult (> 10 weeks) male C57Bl/6 mice were used. They were kept under standard housing regulations at the local institution (12:12 hour light cycle, air exchanges, standard diet, 22.5 °C).

The animal facility in Sheffield employs specific pathogen-free (SPF) working conditions which guarantees the absence of a number of defined pathogens. Mice were obtained from the commercial vendors Taconic (previously Harlan, chapter II + IV) and Charles River (chapter II) at 10 weeks of age and kept in reusable plastic cages with a metal grid lid. Animals were allowed to acclimatise in the facility for at least one week before they were sacrificed according to the UK Animals Scientific Procedures Act (1986). Mice were not individually housed for more than one day.

The bioresource facility at SAHMRI also constitutes a SPF facility but animals are kept in individually ventilated cages and staff is required to change into scrubs to minimise pathogen entry. Here, mice from the in house breeding colony were used and this was originally derived from C57Bl/6J mice from the Jackson laboratory. Up to five siblings were kept together in unless mice underwent surgery for tracing experiments or were treated with TNBS to induce colitis, in which case animals were individually housed.

In addition to the in-house colony, mice from the Jackson laboratory were bought into the SAHMRI facility. These mice were obtained from a “cleaner” specific and opportunistic pathogen free room and to maintain the SOPF standard they were kept in a separated room and it was not permitted to enter after contact with standard SAHMRI animals. They also received a standard chow from that was different from standard SAHMRI animals.

Mice were regularly monitored for signs of distress and illness by trained staff. On the day of experiments, they were killed according to local regulations: overdose of isoflurane (5 % in combination with O_2) followed by cervical dislocation (Sheffield) or CO_2 inhalation followed by exsanguination (Adelaide). Thereafter, a mid-line laparotomy was performed to expose the viscera and the intestines from duodenum to distal colon were carefully removed. They were placed into freshly prepared and gassed (95% O_2, 5% CO_2) Krebs solution (composition in mM: NaCl 120, KCl 5.9, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 15.4, glucose 11.5, and $CaCl_2$ 1.2) and further dissected in a silicone-coated petri dish using a microscope (Figure 1).



1 distal small intestinal afferent
2 proximal colonic afferent
3 ceacum
4 proximal colon
5 distal small intestine (ileum)
6 proximal intestine (duodenum)
7 distal colon

Figure 1: Photograph of the whole length intestine after removal from the peritoneal cavity before further dissection. The location of afferent nerves and intestinal segments that were used in the study are indicated.

2 Electrophysiology

2.1 Small intestinal afferent nerve recordings

Mice were humanely euthanized according to local regulations and the peritoneal cavity was cut open along the midline using scissors. The intestine including duodenum and rectum was removed and placed into fresh carbogenated Krebs solution. To remove the ingesta, the intestine was disentangled by cutting alongside the mesenteric attachments and then flushed with fresh Krebs solution using an injection needle placed on a 10 mL syringe. The tip of the needle was covered with plastic tubing to prevent damaging the tissue. A maximum of three segments proximal to the ileocecal junction was prepared for afferent nerve recordings. Each segment was about 3-4 cm long and contained a single mesenteric attachment. The segment was placed into a custom-build organ bath chamber that was maintained at 33-35 °C and continuously perfused with carbogenated Krebs solution (5 mL/min). It was tied to a luminal perfusion system on both ends with surgical suture (US7/0). A tab connected to the tube at the distal end allowed distension of the tissue. When it is closed, fluid inside the tissue accumulates whilst the pressure increases (Figure 2). Pressure measurements were acquired with the help of a pressure transducer element (DTXPlusTM, BD, Singapore) placed in line with the perfusion system and amplified with a the NeuroLog (digitimer) pressure amplifier (NL108). The mesenteric nerve bundle was dissected from the attachment (Nikon, SMZ-1B, Japan) and placed into a suction electrode that is connected to the Neurolog headstage (NL110). The signal was amplified and filtered using NeuroLog AC amplifier (NL104, x10.000) and filter (NL125) with a band width of 100-1000 Hz. Noise was eliminated using Humbug (Quest Scientific). Nerve activity and pressure data were collected (Micro 1401 MKII interface) and transferred to a computer running Spike2 software (version 7.1, Cambridge Electronic Design). The acquired nerve activity was quantified based on shape and amplitude of spikes by a spike processor (Digitimer D130). The threshold was set above the noise level for each experiment individually. Spike2 software was also used for online and offline data analysis.

Nerve activity was recorded continuously and in response to distension. A stable recording was ensured by stabilising the tissue for one hour and performing control distensions. During these, the pressure-induced increase of nerve activity needed to be identical. After two reproducible distensions, stimuli were applied and the tissue continuously distended to 30 mmHg every 15 min to investigate the mechanosensitivity of intestinal afferent nerves (Figure 3).

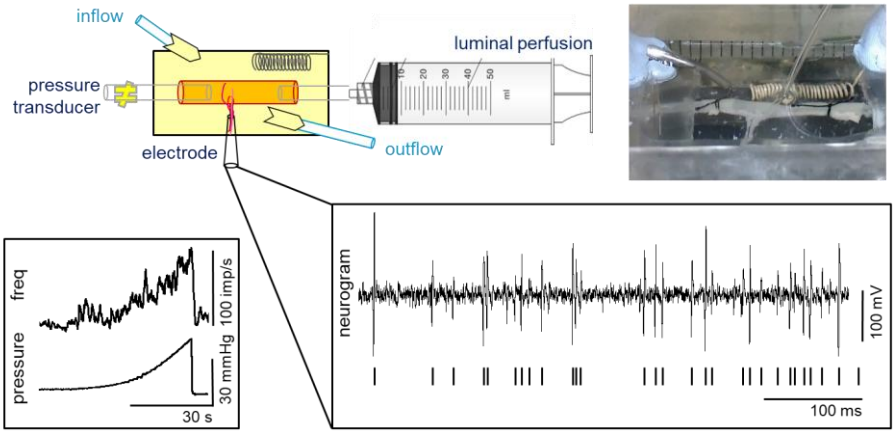


Figure 2: Schematic and photograph of the experimental setup. For description refer to the text.

Spontaneous NA was quantified over 10 min (150 s after the previous distension) and normalised to the firing before the last control distension (% baseline). The response to distension was assessed in intervals of 3 mmHg and the increase of discharge calculated relative to the maximal increase at 30 mmHg which was set to 100 %. For subsequent distensions, mechanosensitivity was expressed relative to the increase of NA at the last control distension ("0 min") with the difference induced by distension to 30 mmHg representing 100 % (% control distension). These analyses were performed using MicrosoftExcel®. Further processing and statistical analysis are stated in the individual chapters.

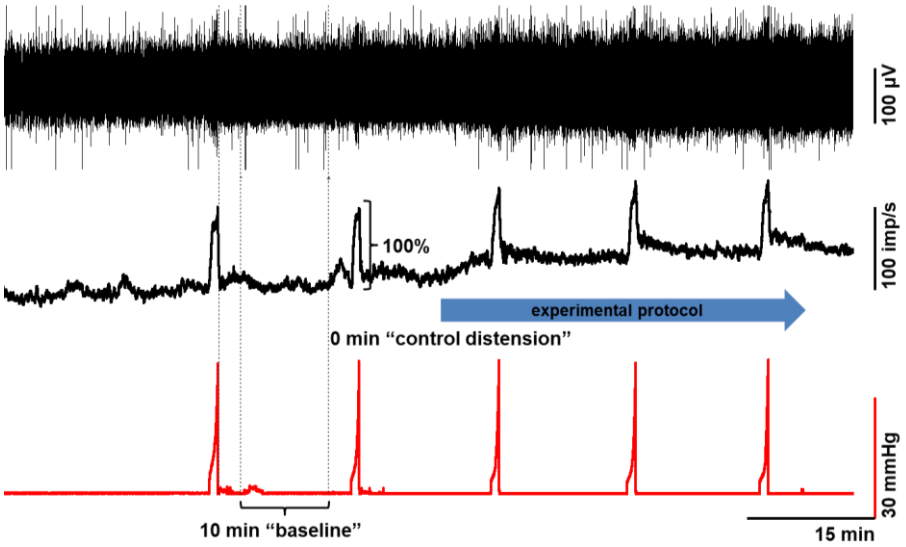


Figure 3: Parameters for data analysis. For analysis of spontaneous firing, the average of a 10 min interval in between two distensions was expressed relative to the NA before the "control distension" at 0 min. Mechanosensitivity was expressed relative to the distension-induced increase of NA at "0 min".

Example trace for bath application of 20 % vehicle: top, neurogram; middle, firing frequency [imp/s]; bottom, pressure

2.2 Colonic motility and afferent nerve recording

Colonic tissue was obtained from male C57Bl/6 mice in order to study the effect of *S. aureus* supernatants on motility. Animals were sacrificed according to legal guidelines, the whole-length intestine was removed after performing a midline laparotomy and placed into carbogenated Krebs solution. The colon was isolated in a Krebs-filled petri-dish and flushed using a tube-covered injection needle attached to a 10 mL injection syringe. In order to prevent damaging the nerves, particular care was taken when the cecum was dissected and faecal pellets were removed through the oral end of the colon. A four-centimetre-long piece of the colon was placed into a custom-made sylgard-covered organ bath chamber and both ends tied to tubes that allow intraluminal perfusion. The fat tissue that is attached to the characteristic curvature of the proximal colon was kept intact and used to carefully pin the tissue. The tube at the anal end of the colon was connected to an outflow tab and pressure transducer through a T piece plastic connector. Tissue was perfused at 200 µL/min during the nerve dissection to prevent accumulation of fluid inside the colon. Nerves innervating the proximal colon were placed into a suction electrode which was connected to the NeuroLog headstage (NL 110). In analogy to the small intestinal recordings, the signal was amplified (NL 104), filtered (NL 125) and acquired (Micro 1401) using Spike2 software.

Spontaneous contractions were induced after maximal 30 min dissection to optimise viability of the tissue and comparability of the data. To induce colonic migrating motor complexes (MMC), the intraluminal perfusion rate was decreased to 100 µL/min and the outflow tab was closed (Keating et al., 2010). This allows to measure the intraluminal pressure by the pressure transducer which is connected to a pressure amplifier in the NeuroLog headstage and the 1401 digitizer. Fluid accumulation was monitored and perfusion stopped when the pressure reached 5 mmHg. Supernatants or vehicle at a concentration of 5 % in Krebs were bath-applied after the first contractions to investigate their effect on the generation of MMC. After 60 min, normal Krebs solution was perfused during a 30 min wash period. Subsequently, the muscarinic Ach receptor agonist Bethanechol (100 µM) was applied to induce contractions of the smooth muscle contraction without directly affecting neuronal activity.

The amplitude and frequency of contractions was analysed during 15 min time intervals at the end of SSA or vehicle application (45-60 min) and the subsequent 30 min wash period (75-90 min). The number of complexes was manually determined and the maximal pressure within the 15 min time periods or during Bethanechol application was used to compared the effects of SSA or vehicle on contraction amplitude.

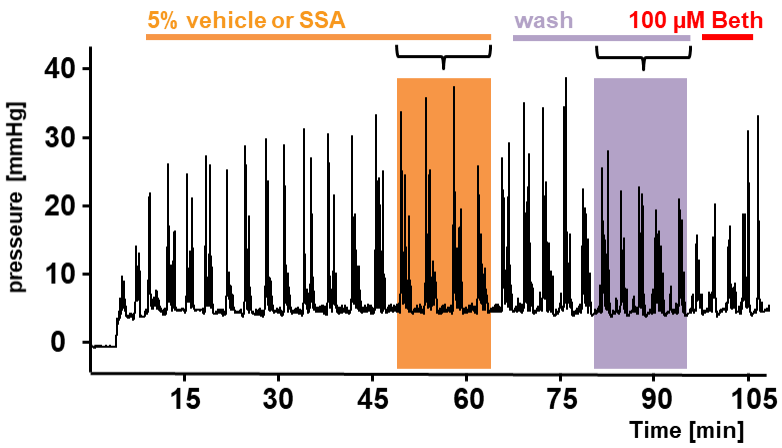


Figure 4: Representative recording of colonic migrating motor complexes during which 5 % vehicle was applied. Data was analysed as described in the text.

3 Bacterial supernatants

Supernatants were prepared from cultures of different *S. aureus* strains and mutants (Table 1) according to standard microbiological procedures and safety precautions. Bacteria were stored on bacterial beads (Microbank™) at -80 °C. For culture, a single beads covered with bacteria was spread onto a nutrient agar plate. Those were prepared from nutrient broth (tryptic soil broth, TSB) that was prepared according to manufacturer's instructions and 1.2-1.5 % bacteriological agar were added. The suspension was autoclaved (120 °C for 15-20 min) and allowed to cool down before pouring into plastic petri dishes under sterile working conditions. Antibiotics (200 µg/mL spectinomycin, 10 µg/mL erythromycin) were added for selection of the Psm and protease mutant (Table 1). Plates were kept in a sterile bag at 4 °C until usage. After plating the bacteria on the plates, they were allowed to grow overnight (16 hours) and moved to 4 °C afterwards. Plates were used for maximal 3-4 weeks.

Table 1: Strains and mutants of *S. aureus* that were used in the current study.

AgrA, accessory gene regulator; aur, aureolysin; CA-MRSA, community-associated multidrug-resistant *S. aureus*; erm, erythromycin, Hla, α-hemolysin; Pvl; Panton-valentine leucocidin; SF, Simon Foster; spec, spectinomycin

*** kindly provided by Angelika Gründling (Imperial College London, SW7 2AZ), ** kindly provided by Micheal Otto (NIAID/NIH Bethesda, MD 20814)**

bacteria	description	reference
JE2 (USA300) - SF strain collection 4276	USA300 constitutes an CA-MRSA strain with repaired antibiotic resistance	(Fey et al., 2013)
Newman - SF strain collection 3662	clinical isolate from a human patient	(Baba et al., 2008)
SH1000 - SF strain collection 682	laboratory derivative of *S. aureus* 8325-4 (RN6390)	(Horsburgh et al., 2002)
SAUSA300_1058 - SF strain collection EF1354	Hla-deficient USA300 mutant from the Nebraska library	(Fey et al., 2013)
SAUSA300_1992 - SF strain collection EF1532	AgrA-deficient USA300 mutant from the Nebraska library	(Fey et al., 2013)
SAUSA300_1382 - SF strain collection EF 1848	Pvl-deficient USA300 mutant from the Nebraska library	(Fey et al., 2013)
AH1919* (USA300 protease KO)	Δaur, ΔsspAB, ΔscpA, spl::erm	(Wörmann et al., 2011)
AH1263*	ermS version of USA300	(Boles et al., 2010)
S. aureus LAC ΔPsmαβhld**	Psm-deficient USA300	(Wang et al., 2007)
S. aureus LAC**	specS version of USA300	(Wang et al., 2007)
endogenous *S. aureus*	isolated from murine intestines using Baird Parker plates	

Individual colonies were picked with a sterile inoculation loop and used to inoculate 50 mL autoclaved TSB in a 250 mL flask. These starter cultures were grown overnight on a rotary shaker at 37 °C. Optical density (OD_{600}) was determined using a spectrometer against nutrient broth and this measurement was used to inoculate 500 mL of pre-warmed broth in a 2 L flask to an OD of < 0.2 (usually 1:100 dilution). These cultures were incubated on a rotary shaker at 37 °C for 24 hours except stated otherwise to ensure that bacteria reached stationary growth phase. Afterwards, optical density was measured and suspension cultures were centrifuged (10 min, > 5000 rpm, 4 °C). Supernatants were aliquoted in 50 mL plastic tubes and stored at -20 °C until usage.

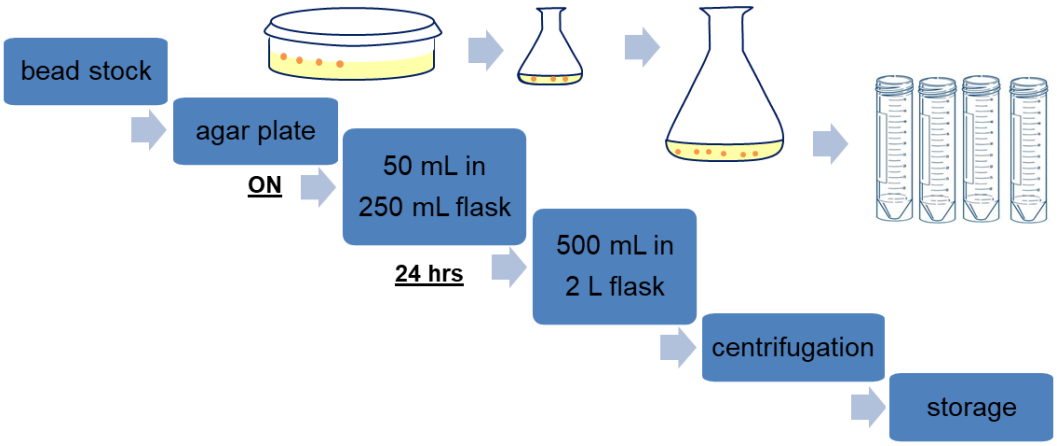


Figure 5: Workflow diagram for preparation of bacterial supernatants.

4 Primary cultures of dorsal root ganglia neurons (Sheffield)

The entire spinal cord was removed from adult male C57BL/6 mice after sacrificing them according to Schedule 1 regulations (UK Animal Scientific procedure Act, 1986). It was cleaned from surrounding muscle tissue and using scissors. DRG were dissected from all spinal levels and collected in ice-cold PBS in a small petri dish. The processes were cut under a dissection microscope and the isolated DRG transferred into 1 mL Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) with Glutamax medium (Gibco) that contained Dispase II (1 mg/mL, Sigma) and Collagenase Type XI (0.6 mg/mL, Sigma). Enyzmes were dissolved in PBS as 100x solutions, diluted in DMEM/F12, aliquoted (1 mL) and stored at -20 °C until usage.

DRG in DMEM/F12 (+enzymes) were incubated for 60 min at 37 °C and 5% CO_2. Then, they were triturated using a P1000 pipette (10-15 time) and the cell suspension carefully layered onto 15 % Bovine Serum Albumin (Melford) in PBS. After centrifugation (800 g, 10 min, room temperature, slow brakes), the supernatant was discarded and the cell pellet was washed with 2 mL culture medium (DMEM/F12 with 10 % Foetal Bovine Serum [FBS, Gibco], 1 % penicillin and streptomycin [Gibco]). Cells were centrifuged again (1200 g, 5 min, room temperature), re-suspended in a small volume (< 250 µL) culture medium and 20 µL plated directly onto tissue culture-treated 24 well plates (Corning™). One hour after plating, cells were flushed with 1.5 mL tissue culture media and used for propidium iodide experiments within 48 hours.

5 Expression analysis

We assessed the expression of genes with described roles in the pathogenesis of *S. aureus* in intestinal tissue, dorsal root ganglia and isolated neurons using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Tissue samples and cells were obtained from healthy C57Bl/6 animals kept in the animal facility of the SAHMRI. Animals were humanely killed and dorsal root ganglia from intestine-innervating regions of the spinal cord (T10-T13 and L5-S1) as well as the nodose ganglia were isolated as previously described. Additionally, ganglia from upper regions of the spinal cord (CT8) which do not contain many intestine-innervating neurons were isolated to investigate selective expression of target genes. The distal intestine proximal to the ileocecal junction was removed and cleaned as described for the afferent nerve recordings. In addition to a whole thickness segment (2-3 cm), the muscle layer and mucosa were separated under the dissection microscope to assess gene expression in these layers of the intestinal wall separately. All samples were frozen in PBS (-80 °C).

5.1 RNA isolation

RNA from these samples and individual visceral neurons which were collected after cultivation of DRG from animals that were ip-injected with CTB-488 4 d prior to the experiment, was isolated in a designated RNA room to prevent degradation and contamination. Samples were kept on ice at all times.

The PureLink® RNA Mini kit was used according to manufacturer's instructions for RNA isolation from intestinal tissue. Eppendorf tubes with lysis buffer (+ DTT) and metal ball were prepared and weighted. Tissue was defrosted on ice, added into the tubes and weighted again. The amount of lysis buffer was adjusted and tissue disrupted in a cooled tissue homogeniser for 40 s. Sample was centrifuged and supernatant mixed with the same volume of 70 % ethanol in a fresh RNAse-free tube. A maximum of 700 µL was transferred to the Spin Cartridge and centrifuged (12.000 g, 15 s) to bind the RNA to the column. The flow-through was discarded and the procedure repeated with additional lysate. The column was washed (washing buffer I, 2x washing buffer II) by pipetting them to the Spin Cartridge and centrifuging afterwards (12.000 g, 15 s). Column was dried by centrifugation (12.000 g, 1 min) and incubated with DNAse to degrade potentially contaminating DNA. DNAse treatment was stopped and RNA removed from the column by adding 20 µL RNAse-free water and centrifugation (12.000 g, 30 s). This step was repeated to increase RNA yield. RNA was stored at -80 °C.

RNA from whole ganglia and single cells was isolated using NucleoSpin® RNA XS kit (Machenery-Nagel™). Whole ganglia were added to Eppendorf tubes prepared with metal ball and RA1 buffer (+ TCEP). The amount of RA1 was adjusted to the weight as described above. Ganglia were lysed in a tissue homogeniser (40 s). Single cells had been collected in 10 µL RA1 (+ TCEP) and 20 lysates were combined prior for RNA isolation. Carrier RNA was added to lysates from whole ganglia and single cells to increase the RNA yield. They were filtered and mixed with equal amounts 70 % ethanol. This was loaded to the silica membrane and bound by centrifugation (11.000 g, 30 s). Flow-though was discarded, membrane desalting buffer (MDB) added and centrifuged (11.000 g, 30 s). DNA was digested and membrane was washed twice with washing buffer RA2 and washing buffer RA3 (11.000 g, 2 min). RNA was eluted after incubating the membrane with 10 µL RNAse-free water in to a fresh Eppendorf tube. We repeated this step to increase the amount of eluted RNA and stored RNA at -80 °C.

For all samples, RNA yield and quality was determined using NanoDrop. The amount of RNA varied considerably between samples but was of good quality. However, readings for some samples obtained from single cells were outside recommended boundaries which is indicative of RNA concentrations at the detection limit. As there is considerable dispute about the meaningfulness of RNA-quantification using NanoDrop™, we continued to process those samples in qRT-PCR experiments.

5.2 Quantitative reverse transcription PCR

Quantification of RNA expression was performed using EXPRESS One-Step SuperScript® qRT-PCR Kits. This enables fast and consistent reverse transcription of RNA by SuperScript® III Reverse Transcriptase and subsequent cDNA amplification (Platinum® *Taq* DNA Polymerase) in a single reaction and is particularly beneficial for samples with small RNA amounts.

Reactions were prepared in a designated PCR room and at 4 °C using iceless cooling plates. Master-mixes were prepared whenever possible to minimise pipetting errors. SuperMix Universal, water and the SuperMix enzymes were mixed and split according to the number of genes of interest. To each tube, the primer/probe mix for the gene of interest was added. 15 µL of this MasterMix were pipetted into the qRT-PCR plate and 5 µL RNA were added. The final composition of the 20 µL reactions was 10 µL EXPRESS SuperScript® qPCR SuperMix Universal, 2 µL water, 2 µL EXPRESS SuperScript® Mix for One-Step qPCR, 1 µL TaqMan assay and 5 µL RNA. The RNA input for single neurons and whole ganglia was 20 ng/reaction whereas preliminary experiments indicated that 200 ng/reaction gave more

consistent results for intestinal tissues. The plate was sealed with a plastic film and processed using standard cycling protocols (15 min 50 °C cDNA synthesis, 95 °C 20 s initiation, 45 cycles 4 s 95 °C and 30 s 60 °C amplification) using AB7500 qRT-PCR machines (AppliedBiosience).

We used the commercially available and validated TaqMan® assays listed in Table 2. They are composed of unlabelled forward and reverse primers and a probe with the fluorescent FAM™ dye at the 5' and a quencher as well as a minor groove binder on the 3' end. As long as the probe is bound to the cDNA transcript, FAM™ fluorescence is quenched. When DNA polymerase synthesises new transcripts the dye is cleaved and emits fluorescence. The more DNA is synthesised the higher is the signal and increases above background fluorescence after a sample-specific number of polymerisation cycles (CT value). This CT value is dependent on the amount of starting material and the abundance of a gene within a sample. To account for differences of starting template and thus, allow comparisons between samples, CT values of genes of interest are normalised to CT values of housekeeping genes (ΔCT). Because the expression housekeeping genes is not consistent between different tissues, it is suggested to use the geometric mean of several housekeeping genes for normalisation.

Table 2: TaqMan® assays used for assessment of gene expression.

assay	gene	protein	Catalogue#
Mm00545742_m1	Adam10	A Disintegrin and metalloproteinase domain-containing protein 10	4453320
Mm00522643_m1	Sgms1	Sphingomyelin Synthase 1	4448892
Mm00442803_s1	Fpr1	Formyl Peptide Receptor 1	4453320
Mm00484464_s1	Fpr2	Formyl Peptide Receptor 2	4448892
Mm01962454_s1	Fpr3	Formyl Peptide Receptor 3	4448892
Mm99999915_g1	Gapdh	Glyceraldehyde-3-Phosphate Dehydrogenase	4331182
Mm00607939_s1	β-actin		4331182

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